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The gene encoding the neu/erB-2/HER-2 receptor tyrosine kinase is one of a small number of genes known to be altered in human breast and ovarian carcinoma. It is amplified and /or over expressed in approximately one-fourth of these cancers. With its probable role in etiology, and expression at the cell surface, neu has great potential as a prognostic indicator and therapeutic target. Efforts to exploit this receptor in the clinical arena have been impeded by the failure to consider regulation of neu by hormones and co-receptors. this has been complicated by the fact that at least seven different peptide hormones, epidermal growth factor (EGF), transforming growth factor-;alpha, amphiregulin, betacellulin, heparin-binding EGF-like growth, epiregulin, and the diverse neuregulins are all able to activate neu. They work by binding to the co-receptors EGF receptor, erB-3 and erB-4, which then dimerize with neu. Work by this laboratory has shown that each one of these factors activ ates a different constellation of receptors, leading to a great diversity of possible responses. Moreover, the coupling of cellular responses to each factor is determined by the subset of receptors expressed in each tissue. Thus in order to interpret the function of any one of the factors or receptors, including neu, it is essential to consider the influences provided by the entire set.

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FOREWORD

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INTRODUCTION

Breast cancer is one of the most important cancers afflicting women and presents challenging treatment decisions. After p53 alterations, the most frequent change in an identified gene is amplification and/or overexpression of the *neu/erbB-2/HER-2* gene, which occurs in up to one-third of breast cancers. The gene product (denoted p185 or non-italicized neu), is a receptor tyrosine kinase (RTK) [1, 2, 3]. This gene was originally discovered in mutant form in chemically-induced rat nervous system tumors and is now known to be a member of the Type 1, or epidermal growth factor (EGF) receptor gene (*erbB*) family. The family includes four receptors, which will be referred towill be referred to herein as the EGFR, neu, erbB-3, and erbB-4. (See our review, [4]). Small-scale screens for *neu* alterations in human tumors led to discovery of changes in a number of adenocarcinomas including breast [5], ovarian, gastric, bladder, lung, and colon. Two influential studies showed that *neu*, and not a panel of other oncogenes is amplified in breast and ovarian carcinomas and that this amplification correlates with RNA and p185 overexpression [6, 7]. Numerous studies of *neu* in mammary carcinoma have led to the following conclusions [4]:

1.*neu* is amplified in 20-30% of mammary carcinomas, with the frequency of amplification higher in tumors from patients with affected lymph nodes [8, 9, 10, 11, 12, 13, 14, 15, 16]. The gene amplification suggests that there is a selection in the tumors for *neu* overexpression. (It cannot be absolutely ruled out that neighboring genes are selected for amplification.)

2.Amplification correlates well with concomitant RNA and p185 expression [6, 7]. An additional 5% of specimens overexpress the receptor without obvious changes in gene structure or copy number [5, 11, 15, 17].

3.There is no evidence for structural mutations in p185 in human tumors. This negative result is weak owing to the high copy number of genes and the large size of the

mRNA. Recent work in the mouse transgenic system suggests that this issue should be reconsidered [18].

4.Amplification and/or p185 overexpression can be found in all grades and stages of carcinomas, but not hyperplasia or dysplasia. It is found more frequently in ductal carcinoma in situ (DCIS) than in infiltrating ductal carcinoma (IDC)[14, 16, 19, 20].

5.Amplification and/or p185 overexpression is associated with poor prognosis, especially in node-positive patients. However the extent of this association and independence from other prognostic markers varies greatly among different studies (reviewed, [4]).

Taken together, these data indicate that *neu* amplification and overexpression play a major role in mammary carcinogenesis. This is consistent with findings in model systems: i) the mutated rat *neu* oncogene is as potent as any including *ras* in tissue culture systems. ii)In contrast to other growth factor receptors including the EGFR, overexpression of p185 in the absence of ligand is sufficient to transform cells [21, 22]. iii)Transgenic mice harboring a mutationally activated *neu* oncogene develop multi-focal mammary carcinoma when expressed under control of a murine mammary tumor virus (MMTV) promoter, which confers high level expression in mammary gland and a few other tissues (not found in all studies [23, 24]. iv)Perhaps most compelling is the fact that transgenic mice carrying a structurally normal *neu* gene driven by the MMTV promoter develop metastatic mammary carcinomas [25]. This is noteworthy because it reconstructs what appears to be occurring in human cancer: overexpression of normal p185 in mammary tissue.

Since p185^{neu} is a cell surface protein that seems to play a causal role in mammary carcinogenesis, it is under intensive investigation as a therapeutic target [4, 26]. Phase II clinical trials, in which patients were infused with anti-neu antibody 4D5 have been completed with a roughly 15% response rate, and represent the vanguard for expanded therapeutic trials targetting this receptor (J. Baselga, personal communication). In spite of

the findings linking *neu* to mammary carcinoma, and despite the fact that patients are already being exposed to neu antagonists, little is known about the function of neu either in the organism, or in breast cancer.

The physiological function of p185*neu*, like any hormone receptor, can only be understood in the context of the hormones that regulate it. The EGFR is activated by binding of at least six different peptide hormones, EGF, TGF-α, amphiregulin (AR), betacellulin (βC), epiregulin [27](epi) and heparin-binding EGF-like growth factor (Hb-EGF)[28, 29, 30, 31]. p185, by itself, cannot bind or be activated by these hormones (epi has not been tested). We discovered that EGF and TGF-α, which do not bind to p185, activate p185 Tyr phosphorylation and stimulate p185-associated kinase activity [2, 32]. This phenomenon, now termed **transmodulation**, is dependent upon the co-expression of the EGF receptor with p185 [33, 34, 35, 36]. It probably occurs at least in part through formation of receptor heterodimers [37, 38]. Transmodulation of neu by the EGFR is biologically relevant since it works with EGF, TGF-α, betacellulin (see below) and AR, stoichiometrically activates p185 [32], permits association of substrates [39], and correlates with *in vivo* synergy in transforming ability of these two receptors [40]. Thus wherever the two receptors are co-expressed, EGFr agonists activate *neu* . In cell lines that express both receptors, EGF-regulated neu signaling is at least as important as signaling by the EGFR [41]. Since p185 and the EGFR have distinguishable signaling activities [42], this means that regulation of neu production provides a means to alter the signal coupled to EGF.

The transmodulation of neu by the EGFR is a prototype for other interactions within the Type 1 receptor family discovered more recently. Ignorance of these interactions has confused many groups studying the EGFR and resulted in a rather muddy literature which is just now being rationalized [4, 43]. For example, several laboratories independently identified an activity termed Heregulin, neu diffentiation factor, gp30, p75, neuregulin, ARIA, and Glial Cell Growth Factor (GGF) [44, 45, 46, 47, 48, 49, 50, 51], a family of related proteins evidently produced by alternate splicing [50](They

will be referred to collectively here as NRG, for the composite name neuregulin, or as NDF). At first the NRGs seemed to be neu ligands since they activate p185 tyrosine phosphorylation in the absence of the EGFR and could be cross-linked to neu [45, 49]. However, it is now known that NRGs bind to both erbB-3 and erbB-4 which can then activate neu by transmodulation [52, 53]. A further complication of this receptor system is that erbB-3 lacks robust kinase activity, and itself requires a second receptor for activity [54].

Additional candidates for neu ligands have been identified but not yet expressed in recombinant form and tested for activity [55, 56]. Nonetheless, the independent purification of NRGs by three different laboratories seeking the *neu* ligand suggests that in mammary epithelia the significant inputs to *neu* may come through transmodulation: transmodulating agonists TGF-α, AR, and NDFs are often produced in mammary tissue or cell lines [57, 58, 59] as are the cooperating receptors. Even if these hormones are uniquely responsible for neu activation in mammary tissue, the biological complexities may be enormous. TGF-α and AR, although both EGFR agonists, have somewhat different biological activities [28]. NRGs at first seemed to have radically different activities than EGF agonists since they promote differentiation in some cell lines [49, 60] (but not others [45, 50]), but this has still not been verified in tissue [4].

In summary, *neu* amplification and overexpression is likely to play a significant role in carcinomas where it occurs. However, the presence of activating mutations and agonistic peptide hormones will regulate *neu* function much more strongly than abundance. The Type 1 RTKs comprise a network in which the signaling potential of each receptor is conditioned not only by the presence of hormones, but is further regulated by the co-expression of related RTKs. The long-term focus of this grant is to define the capabilities of the erbBfamily receptor network: the spectrum of hormones that activate each receptor and receptor combination, and the differences among signalling pathways governed by these receptor systems. These objectives include:

Aim 1: Signalling of individual receptors and receptor combinations will be compared by investigating receptor phosphoryaltions and substrate phosphorylations to determine how receptor interactions modulate signalling specificity.

Aim 2: Functions of NRG ecto- and endo-domains will be analyzed.

Aim 3: Biological activity of NRGs and NRG/TGF-α combinations will be determined in tissue culture.

BODY

ErbB Receptor Signaling Network

This has been an exciting year for the ErbB receptor signaling network, since the first ErbB2-directed therapy performed well in Phase III clinical trials and has been approved by the FDA for treatment of breast cancer patients. As discussed in last year's progress report, we have completed the intended work for Aims 1 and 3. This work was capped off by publication of our final papers in this series,[66,67]. Besides tidying up final experiments for the latter papers, we have devoted most effort this year on Aim II, NRG Intracellular Domain.

II.NRG Intracellular Domain

The cytoplasmic domain of NRG family EGF-related growth factors is unusual in showing extraordinary diversity of regulation by splicing, and in that some forms have unusually long cytoplasmic tails of unknown function (over 400 amino acids). We hypothesize that these tails are likely to themselves transmit signals so that binding of NRG ecto-domains to the cognate receptors results in bidirectional signaling.

Task 3b. The major focus of work on the NRG intracellular domain has been to develop physical and two-hybrids screens to identify binding partners. We have now nearly completed the screen using the first cytoplasmic exon of NRG as bait. Approximately 4 X 10^6 transformants were tested, yielding 36 positives that fulfilled our initial and final screening criteria. Two-thirds of these have been sequenced and analyzed by comparison to the nucleci acid and protein data bases. The results were as follows:

Amphysin II. Isolated twice.

Enigma.

Myd 88.

4 ribosomal phosphoproteins. These are often encountered as false positives in two hybrids screens.

alpha-Globin. Isolated three times.

β-globin. Isolated three times.

mouse acid phospho-protein.

β-actin

8 different isolates that do not match with known gene sequences.

Triage of positives. Once the remaining positives have been sequenced, it will be possible to produce our final rank-ordered list. Provisionally, we have decided to place greatest emphasis on characterization of Amphyphysin II and Enigma. This is based on the rationale that the ribosomal phospho-proteins are typical false-positives for two hybrids screens, and that globins and actin are highly expressed, and are likely to be over-represented in the two hybrids library. We are very interested in Amphiphysin II and Enigma, which both have the appropriate intracellular localization and biological activities to be plausibly connected with the cytoplasmic domain of NRG.

Amphiphysin II is a recently identified cytoplasmic protein related to Amphiphysin I. Amphiphysin I binds to dynamin, and has been implicated in vesicle endocytosis and recycling in the nervous system [68]. Both Amphiphysin I and II are broadly distributed. They form heterodimers that seem to be important for clathrin-mediated endocytosis[69]. Hence one hypothesis would be that interaction with Ampiphysin II interactions with NRG are related to ligand or kinase C-regulated endocytosis of NRG. Other information about Amphiphysin II suggests possible links with signal transduction. Amphiphysin II contains an SH3 domain, a constituent of many signaling adaptor proteins. Moreover, amphiphysin II has been found to associate with two proto-oncogene products, Myc and Abl[70,71]. Finally, the assembly of amphyphysin II into complexes associated with the cytoskeleton suggests other possible roles in signaling[70,71].

Enigma is a LIM-domain containing protein that binds to Tyrosine-containing motifs in receptor tyrosine kinases. Enigma binding is required for function of the receptor kinase RET[72]. Interestingly, a second LIM-domain containing protein, LIM

kinase, has recently been found by another lab to interact with the NRG cytoplasmic domain[73]. Hence NRG may generate intracellular signals through recruitment of LIM-containing proteins.

In order to characterize these potential interactions further, we have constructed epitope-tagged versions of Amphyphysin II and NRG for transient and stable cell lines. We can detect high level expression of both proteins, and are now determining whether they co-localize or co-immunoprecipitate. Enigma will be worked up the same way. Provided either one of these proteins shows evidence for in vivo interactions, we will continue by mapping the sites of interaction, and correlating mutant activities with changes in function associated with these two candidates, as discussed in the original proposal.

We are also continuing to sequence the remaining group. If time permits, and if Enigma and Amphyphsin II do not pan out, we will epitope tag and test any unmatched open reading frames for association with NRG in transient expression experiments.

Glutathione-S-transferase (GST)-fusion proteins. A complementary method for identification of interacting proteins is affinity purification. This has the advantage of using full-length cellular proteins, that are expressed in their native environment, but probably requires stabler interactions than two-hybrids screening. We are using glutathione agarose affinity purification to identify proteins that bind specifically to GST-fusions of NRG cytoplasmic segments. Once positives are identified, this approach can be used for purification for direct sequencing, to verify positives identified in two-hybrids screens, and/or to verify positives from solid phase screening (see below).
GST fusion proteins have been produced with GST-coding sequences fused to the three intracellular exons. These proteins have been labeled by in vitro phosphorylation at a protein kinase A acceptor site tag, and used as probes in Far-Westerns. So far, these assays have been noisy, and we are working to clean up the background using an irrelevant WW domain fusion protein as negative control.

The same fusion proteins have been used to make GST-fusion protein affinity columns. The biggest technical problem has been non-specific protein binding, which we are working to circumvent. In the long run, if the Far-Western's can be improved, we will use the probe to screen a cDNA library, as discussed in the proposal. Alternatively, clean results in the affinity purification may provide us with material that can be identified using MALDI-Mass Spectrometry.

CONCLUSIONS

In year 4 we have identified candidate interacting proteins for the NRG cytoplasmic domain. Two of these candidates promise to provide an entry into our hypothesized functions for the NRG kinase domain. Identification of any cytoplasmic function for NRG will help understand the means by which this important signaling molecule modulates cellular functions.

REFERENCES

1. Bargmann, C. I., Hung, M. C., and Weinberg, R. A.: The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 1986, 310:226-230
2. Stern, D. F., Heffernan, P. A., and Weinberg, R. A.: p185, a product of the *neu* proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol. Cell. Biol. 1986, 6:1729-1740
3. Coussens, L., Yang-Feng, T. L., Liao, Y.-C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A.: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 1985, 230:1132-1139
4. Hynes, N. E., and Stern, D. F.: The biology of *erbB-2/neu/HER-2* and its role in cancer. Biochemica et Biophysica Acta Reviews on Cancer 1994, 1198:165-184
5. King, C. R., Swain, S. M., Porter, L., Steinberg, S. M., Lippman, M. E., and Gelmann, E. P.: Heterogeneous expression of *erb*B-2 messenger RNA in human breast cancer. Cancer Res. 1989, 49:4185-4191
6. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L.: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 1987, 235:177-182
7. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F.: Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 1989, 244:707-712
8. Ali, I. U., Campbell, G., Lidereau, R., and Callahan, R.: Amplification of c-*erb*B-2 and aggressive human breast tumors? Science 1988, 240:1795-1796
9. Anbazhagan, R., Gelber, R. D., Bettelheim, R., Goldhirsch, A., and Gusterson, B. A.: Association of c-erbB-2 expression and S-phase fraction in the prognosis of node positive breast cancer. Ann. Oncol. 1991, 2:47-53
10. Gasparini, G., Gullick, W. J., Bevilacqua, P., Sainsbury, R. C., Meli, S., Boracchi, P., Testolin, A., La Malfa, G., and Pozza, F.: Human breast cancer: prognostic significance of the c-*erb*B-2 oncoprotein compared with epidermal growth factor receptor, DNA ploidy, and conventional pathologic features. J. Clin. Oncol. 1992, 10:686-695
11. Hynes, N. E.: Amplification and overexpression of the erbB-2 gene in human tumors: its involvement in tumor development, significance as a prognostic factor, and potential as a target for cancer therapy. Sem. in Cancer Biol. 1993, 4:19-26
12. Kallioniemi, O.-P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L.-C., Smith, H. S., Waldman, F. M., Pinkel, D., and Gray, J. W.: *ERBB2* amplification in breast cancer analyzed by fluorescence *in situ* hybridization. Proc. Natl. Acad. Sci. USA 1992, 89:5321-5325

13. Levine, M. N., and Andrulis, I.: The Her-2/*neu* oncogene in breast cancer: so what is new? J. Clin. Oncol. 1992, 10:1034-1036

14. van de Vijver, M. J., Peterse, J. L., Mooi, W. J., Wisman, P., Lomans, J., Dalesio, O., and Nusse, R.: *Neu*-protein overexpression in breast cancer: Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N. Engl. J. Med. 1988, 319:1239-1245

15. Clark, G. M., and McGuire, W. L.: Follow-up study of HER-2/*neu* amplification in primary breast cancer. Cancer Res. 1991, 51:944-948

16. Allred, D. C., Clark, G. M., Molina, R., Tandon, A. K., Schnitt, S. J., Gilchrist, K. W., Osborne, C. K., Tormey, D. C., and McGuire, W. L.: Overexpression of HER-2/*neu* and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. Hum. Pathol. 1992, 23:974-979

17. Kraus, M. H., Popescu, N. C., Amsbaugh, S. C., and King, C. R.: Overexpression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO 1987, 6:605-610

18. Siegel, P. M., Dankort, D. L., HArdy, W. R., and Muller, W. J.: Novel activating mutations in the *neu* proto-oncogene involved in induction of mammary tumors. Molecular and Cellular Biology 1994, 14:7068-7077

19. Paterson, M. C., Dietrich, K. D., Danyluk, J., Paterson, A. H. G., Lees, A. W., Jamil, N., Hanson, J., Jenkins, H., Krause, B. E., McBlain, W. A., Slamon, D. J., and Fourney, R. M.: Correlation between c-*erb*B-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res. 1991, 51:556-567

20. Paik, S., Hazan, R., Fisher, E. R., Sass, R. E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M. E., and King, C. R.: Pathologic findings from the national surgical adjuvant breast and bowel project: prognostic significance of *erb*B-2 protein overexpression in primary breast cancer. J. Clin. Oncol. 1990, 8:103-112

21. Hudziak, R. M., Schlessinger, J., and Ullrich, A.: Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA 1987, 84:7159-7163

22. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A.: *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 1987, 237:178-182

23. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P.: Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. Cell 1988, 54:105-115

24. Bouchard, L., Lamarre, L., Tremblay, P. J., and Jolicoeur, P.: Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-*neu* oncogene. Cell 1989, 57:931-936

25. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J.: Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proceedings of the National Academy of Sciences USA 1992, 89:10578-10582

26. Baselga, J., and Mendelsohn, J.: Receptor blockade with monoclonal antibodies as anti-cancer therapy. Pharmac. Ther. 1994, 64:127-154

27. Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K.: Epiregulin: A novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. Journal of Biological Chemistry 1995, 270:7495-7500

28. Plowman, G. D., Green, J. M., McDonald, V. L., Neubauer, M. G., Disteche, C. M., Todaro, G. J., and Shoyab, M.: The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. Mol. Cell. Biol. 1990, 10:1969-1981

29. Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K., and Folkman, J.: Betacellulin: a mitogen from pancreatic beta cell tumors. Science 1993, 259:1604-1607

30. Derynck, R.: The physiology of transforming growth factor-alpha. Adv. Cancer Res. 1992, 58:27-52

31. Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C., and Klagsbrun, M.: A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251:936-939. Science 1991, 251:936-939

32. Stern, D. F., and Kamps, M. P.: EGF-stimulated tyrosine phosphorylation of p185^{neu}: a potential model for receptor interactions. EMBO Journal 1988, 7:995-1001

33. Connelly, P. A., and Stern, D. F.: The epidermal growth factor receptor and the product of the *neu* protooncogene are members of a receptor tyrosine phosphorylation cascade. Proc. Natl. Acad. Sci. USA 1990, 87:6054-6057

34. King, C. R., Borrello, I., Bellot, F., Comoglio, P., and Schlessinger, J.: EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the *erb*B-2 protein in the mammary tumor cell line SK-BR-3. EMBO 1988, 7:1647-1651

35. Johnson, G., Kannan, B., Shoyab, M., and Stromberg, K.: Amphiregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and p185erbB2. Evidence that amphiregulin acts exclusively through the epidermal growth factor receptor at the surface of human epithelial cells. J. Biol;. Chem. 1993, 268:2294-2931

36. Spivak-Kroizman, T., Rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J., and Lax, I.: Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits stimulatory or inhibitory responses. J. Biol. Chem. 1992, 267:8056-8063

37. Goldman, R., Ben Levy, R., Peles, E., and Yarden, Y.: Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochem. 1990, 29:11024-11028

38. Wada, T., Qian, X., and Greene, M. I.: Intermolecular association of the p185^{neu} protein and EGF receptor modulates EGF receptor function. Cell 1990, 61:1339-1347

39. Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y.: Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests compolex ligand-receptor relationships. EMBO Journal 1993, 12:961-971

40. Kokai, Y., Meyers, J. N., Wada, T., Brown, V. I., LeVea, C. M., Davis, J. G., Dobashi, K., and Greene, M. I.: Synergistic interaction of p185 c-*neu* and the EGF receptor leads to transformation of rodent fibroblasts. Cell 1989, 58:287-292

41. Graus-Porta, D., Beerli, R. R., and Hynes, N. E.: Single-chain antibody-mediated intracellular retention of erbB-2 impairs neu differentiation factor and epidermal growth factor signaling. Molecular and Cellular Biology 1995, 15:1182-1191

42. Di Fiore, P. P., Segatto, O., Taylor, W. G., Aaronson, S. A., and Pierce, J. H.: EGF receptor and *erb*B-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. Science 1990, 248:79-83

43. Earp, J. S., Dawson, T. L., Li, X., and Yu, H.: Heterodimerization and functional interaction between EGF receptro family members: A new signaling paradigm with implications for breast cancer research. Breast Cancer Research and Treatment 1995, 35:115-132

44. Lemke, G.: Recitative and aria. Nature 1993, 362:291-292

45. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W.-J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L.: Identification of heregulin, a specific activator of p185^{erb}B2. Science 1992, 256:1205-1210

46. Lupu, R., Colomer, R., Zugmaier, G., Sarup, J., Shepard, M., Slamon, D., and Lippman, M. E.: Direct interaction of a ligand for the *erb*B2 oncogene product with the EGF receptor and p185^{erb}B2. Science 1990, 249:1552-1555

47. Lupu, R., Colomer, R., Kannan, B., and Lippman, M. E.: Characterization of a growth factor that binds exclusively to the *erbB-2* receptor and induces cellular responses. Proc. Natl. Acad. Sci. USA 1992, 89:2287-2291

48. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R., and Yarden, Y.: Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell 1992, 69:205-216

49. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y.: Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell 1992, 69:559-572

50. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Berminghan-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D.: Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature 1993, 362:312-318

51. Corfas, G., Falls, D. L., and Fischbach, G. D.: ARIA, a protein that stimulates acetylcholine receptor synthesis, also induces tyrosine phosphorylation of a 185kDa muscle transmembrane *protein.* Proc. Natl. Acad. Sci. USA 1993, 90:1624-1628

52. Plowman, G., Green, J., Culouscou, J.-M., Carlton, G., Rothwell, V., and Buckley, S.: Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. Nature 1993, 366:473-475

53. Sliwkowski, M., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III: Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. Journal of Biological Chemistry 1994, 269:14661-14665

54. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and K L Carraway, I.: Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proceedings of the National Academy of Sciences USA 1994, 91:8132-8136

55. Huang, S. S., and Huang, J. S.: Purification and characterization of the neu/erb B2 ligand-growth factor from bovine kidney. J. Biol. Chem. 1992, 267:11508-11512

56. Dobashi, K., Davis, J. G., Mikami, Y., Freeman, J. K., Hamuro, J., and Greene, M. I.: Characterization of a neu/c-erbB-2 protein-specific activating factor. Proc. Natl. Acad. Sci. USA 1991, 88:8582-8586

57. Normanno, N., Qi, C.-F., Gullick, W. J., Persico, G., Yarden, Y., Wen, D., Plowman, G., Kenney, N., Johnson, G., Kim, N., Brandt, R., Martinez-Lacaci, I., Dickson, R. B., and Salomon, D. S.: Expression of amphiregulin, cripto-1, and heregulin α in human breast cancer cells. Int. J. Oncol. 1993, 2:903-911

58. Liscia, D. S., Merlo, G., Ciardiello, F., Kim, N., Smith, G. H., Callahan, R., and Salomon, D. S.: Transforming Growth Factor-α Messenger RNA Localization in the

Developing Adult Rat and Human Mammary Gland by in Situ Hybridization. Developmental Biology 1990, 140:123-131

59. LeJeune, S., Leek, R., Horak, E., Plowman, G., Greenall, M., and Harris, A. L.: Amphiregulin, Epidermal Growth Factor Receptor, and Estrogen Receptor Expression in Human Primary Breast Cancer. Cancer Research 1993, 53:3597-3602

60. Culouscou, J.-M., Plowman, G. D., Carlton, G. W., Green, J. M., and Shoyab, M.: Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180^{erb}B4 receptor. J. Biol. Chem. 1993, 268:18407-18410

61. Riese II, D. J., van Raaij, T. M., Plowman, G. D., Andrews, G. C., and Stern, D. F.: Cellular response to neuregulins is governed by complex interactions of the erbB receptor family. Mol.Cell.Biol. 1995, 15:5770-5776

62. Riese II, D. J., Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F.: Betacellulin activates the epidermal growth factor receptor and erbB-4 and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β. Oncogene 1996, 12:345-353

63. Riese II, D., Kim, E., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G. D., and Stern, D. F.: The epidermal growth factor receptor couples transforming growth factor alpha, heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB-3, and ErbB-4. Journal of Biological Chemistry 1996, 271:20047-20052

64. Chang, H., Riese II, D.J., Gilbert, W., Stern, D.F., and U.J. McMahan. 1997. Ligands for ErbB family receptors encoded by a newly characterized neuregulin-like gene. Nature: 387: 509-512.

65. Kannan, S., DeSantis, M., Lohmeyer, M., Riese II, D.J., Smith, G.H., Hynes, N., Seno, M., Brandt, R., Bianco, C., Persico, G., Kenney, N., Normanno, N., Martinez-Lacaci, I., Ciardello, F., Stern, D.F., Gullick, W.J., Salomon, D.S. 1997. Cripto enhances the tyrosine phosphorylation of Shc and activates MAP kinase in mammary epithelial cells. J. Biol. Chem. 272: 3330-3335.

66.Riese, II, D.J., Komurasaki, T., Plowman, G.D., and Stern, D.F. Activation of ErbB4 by the bifunctional EGF family hormone epiregulin is regulated by ErbB2. (1998). Activation of ErbB4 by the bifunctional EGF family hormone epiregulin is regulated by ErbB2. J.Biol.Chem. 273, 11288-11294.

67.Riese II, D.J., and Stern, D.F. Specificity Within the EGF Family/ErbB Receptor Family Signaling Network. 1998. BioEssays 20, 41-48.

68.Wigge P, et al. 1998. The amphiphysin family of proteins and their role in endocytosis at the synapse. Trends Neurosci. 21:339-44.

69.Wigge P, et al. 1997. Amphiphysin heterodimers: potential role in clathrin-mediated endocytosis. Mol Biol Cell. 8:2003-15.

70.Sakamuro D, et al. 1996. BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. Nat Genet.14:69-77.

71. Kadlec L, Pendergast AM. 1997. The amphiphysin-like protein 1 (ALP1) interacts functionally with the cABL tyrosine kinase and may play a role in cytoskeletal regulation.Proc Natl Acad Sci U S A 94:12390-5.

72. Durick, K, Gill, G.N., and Taylor, S.S. 1998. Shc and Enigma are both required for mitogenic signaling by Ret/ptc2. Mol. Cell Biol. 18:2298-2308.

73. Jay Y. Wang, Kristen E. Frenzel, Duanzhi Wen, and Douglas L. Falls. 1998. Transmembrane Neuregulins Interact with LIM Kinase 1, a Cytoplasmic Protein Kinase Implicated in Development of Visuospatial Cognition J. Biol. Chem. 273: 20525-20534.

APPENDIX

new publications from the grant from the current project period

Riese II, D.J., and **Stern**, D.F. Specificity Within the EGF Family/ErbB Receptor Family Signaling Network. 1998. BioEssays 20:41-48

Riese, II, D.J., Komurasaki, T., Plowman, G.D., and **Stern**, D.F. 1998. Activation of ErbB4 by the bifunctional EGF family hormone epiregulin is regulated by ErbB2. J.Biol.Chem. 273, 11288-11294.

Specificity within the EGF family/ErbB receptor family signaling network

David J. Riese II and David F. Stern*

Summary

Recent years have witnessed tremendous growth in the epidermal growth factor (EGF) family of peptide growth factors and the ErbB family of tyrosine kinases, the receptors for these factors. Accompanying this growth has been an increased appreciation for the roles these molecules play in tumorigenesis and in regulating cell proliferation and differentiation during development. Consequently, a significant question has been how diverse biological responses are specified by these hormones and receptors. Here we discuss several characteristics of hormone-receptor interactions and receptor coupling that contribute to specificity: 1) a single EGF family hormone can bind multiple receptors; 2) a single ErbB family receptor can bind multiple hormones; 3) there are three distinct functional groups of EGF family hormones; 4) EGF family hormones can activate receptors in *trans*, and this heterodimerization diversifies biological responses; 5) ErbB3 requires a receptor partner for signaling; and 6) ErbB family receptors differentially couple to signaling pathways and biological responses. BioEssays 20:41–48, 1998.

INTRODUCTION

The signaling network composed of the epidermal growth factor (EGF) family of hormones and their receptors regulates the proliferation and differentiation of many tissue types. Deregulation of this network is a significant factor in the genesis or progression of several human cancers, including neoplasms of the brain, lung, breast, ovary, pancreas, and prostate.[1,2] These observations have spurred efforts to elucidate how this signaling network is regulated and coupled to physiological responses and how regulation and coupling are disrupted in malignancies.

Efforts to characterize this signaling network also have been triggered by the observations that the EGF family peptides, called neuregulins (NRGs), play a significant role in neural development and function. Neurons produce NRGs, whereas postsynaptic cells or cells associated with neurons (glia or Schwann cell precursors) express ErbB family receptors. NRG activates these receptors through a paracrine or juxtacrine mechanism. For example, NRGs produced by motor neurons induce acetylcholine receptor subunit transcription and protein synthesis in postsynaptic muscle cells, which express ErbB2 and ErbB3 and possibly EGFR and ErbB4.[3] Furthermore, NRGs expressed from sensory neuron axons stimulate Schwann cell proliferation and may promote the differentiation of neural crest cells into Schwann cell precursors.[4] In general, patterns of ErbB3 and ErbB4 expres-

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sion differ during neural development, suggesting that these NRG receptors couple to distinct signaling pathways and cellular responses.[3] Additional functions for NRGs have been identified in mutants in which NRG signaling is disrupted.[5–8] Such mutants display defects in the peripheral nervous system, most notably a loss of cells in the cranial sensory ganglia or a misinnervation of rhombomeres by cranial sensory and motor neurons. Again, because multiple ErbB family receptors appear to be NRG effectors, there is significant interest in how these receptors couple to distinct signaling effectors and biological function.

EGF FAMILY HORMONES

There are at least eight different hormones in the EGF family: EGF itself, transforming growth factor alpha (TGF-α); heparin-binding epidermal growth factor-like factor (HB-EGF); amphiregulin (AR), also known as keratinocyte autocrine factor or colorectum-cell derived growth factor; epiregulin (EPR); betacellulin (BTC); the neuregulins (NRGs), also known as heregulins, neu differentiation factors, glial growth factors, acetylcholine receptor inducing activity, or sensory and motor neuron-derived factor; and the neuregulin-2s (NRG-2s), also known as the cerebellum-derived growth factors. Moreover, multiple NRG and NRG-2 isoforms arise from alternative transcriptional splicing.[1–4,9–11]

Most EGF family peptides are synthesized as transmembrane precursors that can be proteolytically cleaved to release the soluble form of the hormone or can function as membrane-anchored hormones in juxtacrine signaling. The soluble hormones can be as small as 50 amino acids, sharing a domain of homology that encompasses approximately 50 amino acids. The salient feature of this domain is six characteristically spaced cysteine residues that form three intramolecular disulfide linkages and define a three-loop secondary structure.[2] This domain is both required and sufficient for ErbB family receptor binding and activation; little is known about the physiological functions of the non-EGF homologous domains, which can be extensive.

Two additional proteins share limited homology with EGF family hormones. Cripto-1[12,13] and Cryptic[14] contain the six cysteine residues characteristic of EGF family hormones. However, the spacing of these residues is altered such that Cripto-1 and Cryptic completely lack the "A-loop" formed by the residues between the first and second cysteine residues, and the "B-loop" formed by the residues between the third and fourth cysteine residues is considerably smaller than this domain in other EGF family hormones. Indeed, the synthetic EGF homology domain of Cripto-1 does not activate ErbB family receptors.[15]

ERBB FAMILY RECEPTORS

There are four ErbB family receptors: epidermal growth factor receptor (EGFR, also called HER; ErbB);[16] ErbB2 (also Neu, HER-2);[17,18] ErbB3 (HER3);[19,20] and ErbB4 (HER4).[21] The human forms of these receptors range in size from 1,210 to 1,343 amino acids. They each consist of a cysteine-rich extracellular domain, a single membrane-spanning domain, and a large cytoplasmic domain composed of a tyrosine kinase domain and several tyrosine residues that are phosphorylated upon receptor activation. Ligand binding stimulates receptor dimerization and tyrosine phosphorylation at several sites that then serve to dock effector proteins and couple to physiological responses.

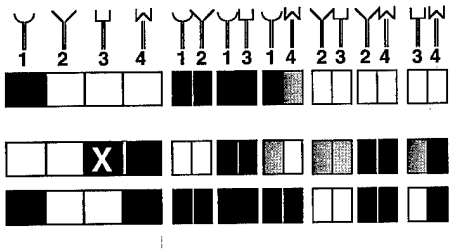

Figure 1. Three distinct functional groups of EGF family hormones. The patterns of ErbB family receptor activation stimulated by various EGF family hormones are shown for cell lines expressing ErbB family receptors singly or in pairwise combinations. Where there are quantitative variations within groups, the pattern shown applies to the prototype member listed first. A filled box indicates receptor activation, with the fill darkness indicating the intensity of receptor activation. A white "x" on a filled background indicates that the hormone binds the receptor but does not activate it. Adapted from references 10, 29, 31, 35, 36, 42, 43, and 74–83.

DIFFERENTIAL ACTIVATION AND COUPLING OF ERBB FAMILY RECEPTORS

Differential Activation of ErbB Family Receptors

A number of mechanisms contribute to the complexity and interconnectedness of the EGF family/ErbB family signaling network. These include the large number of ligands and extensive cross-interactions among the receptors.

(1) A Single EGF Family Hormone Can Bind Multiple Receptors

For example, BTC, HB-EGF, and EPR activate both the EGF receptor and ErbB4, whereas NRG and NRG-2 both bind ErbB3 and ErbB4 (Fig. 1).

(2) A single ErbB Family Receptor Can Bind Multiple Hormones

With the exception of ErbB2, which is an orphan receptor, each ErbB family receptor binds multiple hormones. EGF, TGF-α, HB-EGF, AR, BTC, and EPR bind to the EGFR. BTC,

NRG, NRG-2, HB-EGF, and EPR bind ErbB4, but only NRG and NRG-2 bind ErbB3 (Fig. 1).

(3) There Are Three Distinct Functional Groups of EGF Family Hormones

These groups are distinguished by their abilities to bind to and activate distinct sets of individual receptors (Fig. 1). The first group consists of EGF and its functional analogues TGF-α, and AR, which all bind and activate EGFR but not the other receptors. The second group consists of NRG and NRG-2, which bind ErbB3 and ErbB4. The third group consists of BTC, EPR, and HB-EGF. These hormones bind and activate both EGFR and ErbB4.

(4) EGF Family Hormones Can Activate Receptors in trans

Receptors that do not bind a particular hormone when expressed alone can be cross-activated ("transmodulated") if a binding competent receptor is also present. For example, although EGF does not bind to or activate ErbB2 expressed by itself, EGF induces the tyrosine phosphorylation of both EGFR and ErbB2 in cells expressing both receptors[22–24] (Fig. 1). Furthermore, even a kinase-inactive ErbB2 mutant can be cross-activated by EGF and EGFR.[25] Because the transmodulation of ErbB2 by the EGFR is accompanied by the formation of EGF-stimulated EGFR-ErbB2 heterodimers,[26,27] it is likely that the kinase responsible for ErbB2 transmodulation is the EGFR itself. Nonetheless, a plausible alternative is that an *src*-family kinase activated by the EGFR is involved.[28]

Analogous heterotypic interactions are now known to occur extensively among other combinations of ErbB family receptors (Fig. 1). The presence of a single hormone-binding receptor is generally sufficient for EGF family hormones to activate all other ErbB family members present. However, RTKs outside the ErbB receptor family do not cross-activate these receptors, nor can they themselves be activated in trans by EGF family hormones and ErbB family receptors. There are two notable exceptions. Although BTC activates ErbB4, BTC does not activate ErbB3 in cells expressing both ErbB3 and ErbB4 (Fig. 1).[29] Similarly, although NRG-α binds ErbB3, it does not activate either EGFR or ErbB3 in cells expressing these receptors.[30]

At a more quantitative level, there is a graded hierarchy of heteromeric interactions that may reflect differences in affinities of the various hormone-receptor-receptor complexes. For example, EGF, which binds only the EGFR, transmodulates ErbB2 more strongly than ErbB3 or ErbB4. Similarly, NRG-β, which binds ErbB3 and ErbB4, transmodulates ErbB2 more strongly than EGFR. This suggests that ErbB2 is the preferred target for transmodulation by a ligand-activated ErbB family receptor.[31–33]

Because many cell types express at least three of the four receptors, this implies that there is competition among receptors for dimerization partners. This may explain the finding that pretreatment with NRG inhibits subsequent EGF binding, suggesting that recruitment of the EGFR into complexes with ErbB3 or ErbB4 reduces the availability of free EGFR.[34] Moreover, transmodulation of the EGFR in T47D cells by NRG (via ErbB3 and ErbB4) is enhanced when ErbB2 is selectively removed.[33] However, a simple competition model does not account for the observation that down-regulation of ErbB2 *reduces* ErbB4 transmodulation by EGF (and the EGFR).[33]

The mechanism and stoichiometry of heteromeric receptor interactions have not been determined; this reflects the absence of a basic understanding about receptor oligomerization. For example, it is possible that higher order oligomers rather than dimers are the active signaling species. Furthermore, it is not known if a ligand binds to one or both receptors in a heterotypic receptor complex. Coexpression of binding and nonbinding ErbB family members in some cases enhances hormone-binding affinity,[35] whereas intracellular retention of ErbB2 reduces EGF and NRG binding by accelerating their dissociation.[36] (We use "binding" and "nonbinding" here to denote the behavior of the receptors when expressed individually.) The means by which a nonbinding receptor is recruited into a receptor complex and can modulate ligand-binding affinity are uncertain. Hypothetically, in EGFR/ErbB2 transmodulation, EGF binding may unveil a cryptic EGFR/ErbB2 interreceptor binding site and/or may stabilize EGF binding to ErbB2. Similarly, formation of EGFR-ErbB2 dimers may alter the conformation of the ErbB2 hormone-binding domain or create a composite-binding site encompassing elements from both receptors. EGF appears to bind the EGFR with a 1:1 stoichiometry,[37,38] and it has been proposed that EGF binds bivalently, with each EGF molecule binding to a high-affinity site on one EGFR molecule and a low-affinity site on another EGFR molecule.[38] Bivalent hormone binding is consistent with the observation that BTC and EGF-NRG chimeras bind both EGFR and ErbB4.[29,39] It has been proposed that hormone binding to the high-affinity binding site is required to stabilize receptor dimers and for receptor activation. Therefore, bivalent binding of a hormone molecule to a high-affinity site on one receptor ("binding" receptor) and a lower affinity site on a heterotypic ErbB family receptor molecule ("nonbinding" receptor) may be the mechanism underlying receptor heterodimerization and transmodulation. Differences in the affinity of this "lower affinity" binding site on the "nonbinding" receptor may explain the variable heterodimerization and transmodulation potential for the four ErbB family receptors. And finally, these possibilities mean that coexpression of ErbB family receptors may create novel "emergent" binding specificities.

Intriguing observations that have led to a greater understanding of ErbB family receptor heterodimerization are that

NRG induces the formation of EGFR-Neu heterodimers and that this heterodimerization is blocked by the Neu tyrosine kinase inhibitor tyrphostin AG879. Ligand-induced ErbB family receptor dimers apparently dissociate and can nucleate "secondary" (hetero)dimerization with additional receptor molecules. Moreover, receptor phosphorylation is apparently required for dissociation of the "primary" receptor dimers and for formation of "secondary" heterodimers.[40] Because receptor phosphorylation also triggers receptor internalization and degradation, the amount of receptor heterodimerization must be regulated by a number of factors, including hormone and receptor concentration, the affinity for receptor dimerization, receptor kinase activity, and rate of receptor internalization.

(5) ErbB3 Requires a Partner

A special case is the reliance of ErbB3 signaling on heteromeric interactions. Four residues in the ErbB3 tyrosine kinase homology domain diverge from the consensus tyrosine kinase sequence,[20,41] and ErbB3 has little or no associated kinase activity.[41] Although ErbB3 expressed alone binds NRGs, tyrosine phosphorylation of this receptor only occurs in the presence of additional ErbB family members. Presumably, they are required for cross-phosphorylation of ErbB3.[29,35,36,42,43]

COUPLING OF ERBB FAMILY RECEPTORS TO BIOLOGICAL RESPONSES AND SIGNALING EFFECTORS

The complex regulation of ErbB family receptor activation is significant because the four receptors couple to distinct sets of signaling effectors and biological responses.

(1) ErbB Family Receptors Have Different Signaling Specificities

Each receptor has unique signaling specificities, as first suggested by the unique hormone-independent transforming activity of overexpressed ErbB2 relative to the other receptors.[44–51] These differences reflect differential activation of signaling pathways, as was first shown to occur for EGFR and ErbB2.[46,52]

(2) ErbB Family Receptor Heterodimerization Diversifies Biological Responses

An early indication that activation of multiple ErbB family receptors increases response diversity was the finding that EGFR and ErbB2 synergize in transformation of NR6 fibroblasts.[53] Similarly, a subset of receptor combinations promotes hormone-independent or NRG-dependent transformation of NIH3T3 cells.[48,49,51] Interleukin 3 (IL-3)-independent survival or proliferation of a panel of Ba/F3 cells with defined receptor content depends on the specific combinations of

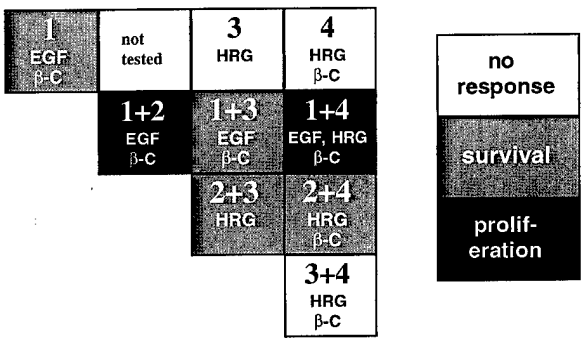

Figure 2. Responses of recombinant Ba/F3 cell lines to ErbB family receptor activation. The patterns of interleukin-3 (IL-3)-independent responses stimulated by various EGF family hormones are shown for Ba/F3 cell lines expressing ErbB family receptors singly or in pairwise combinations. A filled box indicates an IL-3-independent response, with a gray fill indicating IL-3-independent survival and a black fill indicating IL-3-independent proliferation. The hormones used to elicit these responses are indicated by name. Adapted from references 29, 43, and 80.

receptors activated (Fig. 2). In this cell background, activation of ErbB4 alone yields no measurable response, whereas activated EGFR couples to IL-3-independent survival. However, activation of EGFR and ErbB4 together leads to IL-3-independent proliferation.[29,43] Conversely, selective intracellular retention (and inactivation) of ErbB2 in T47D cells radically diminishes the extent and duration of MAP kinase activation by EGF and NRG, suggesting that MAP kinase is predominantly coupled to ErbB2 activated by transmodulation.[36]

The simplest interpretation for receptor cooperativity in induction of biological responses is that different receptors activate complementary signaling pathways. However, an alternative explanation is that receptor heteromers have unique signaling specificities. This is plausible, because in a cross-phosphorylation reaction within a heterodimer, the identity and geometry of kinase/substrate pairs differ from that for a homodimer. In NIH3T3 cells ectopically expressing EGFR and ErbB3, NRG stimulates anchorage-independent growth, whereas EGF does not, despite the fact that EGF stimulates higher levels of receptor phosphorylation than NRG. Furthermore, EGF treatment mobilizes intracellular Ca^{2+} stores, whereas NRG treatment does not.[51] Similarly, when the EGFR is activated by HB-EGF in a breast tumor cell line, the downstream signaling effector Cbl is tyrosine phosphorylated and complexes with the EGFR. However, when EGFR is activated by NRG through transmodulation by ErbB3 or ErbB4, Cbl is not tyrosine phosphorylated, nor does it complex with the EGFR, despite the fact that HB-EGF and NRG stimulate similar levels of EGFR phosphorylation.[33]

These data suggest that distinct sets of receptor tyrosine residues become phosphorylated in response to different stimuli, resulting in differential coupling to signaling effectors and biological responses.

(3) ErbB Family Receptors Couple to Distinct Cellular Signaling Effectors

Although it is clear that ErbB family receptors differentially couple to biological responses, the specific substrates and pathways associated with these different responses have not been elucidated. A number of receptor effectors have been identified, a few of which may be receptor-specific.[1,31,36,46,52,54-59] For example, Cbl is activated by and complexes with the EGFR but not the other ErbB family receptors.[60] Similarly, the CHK Csk-homologous kinase binds to ErbB2 but does not bind EGFR, ErbB3, or ErbB4. [61]

CONCLUSIONS

Emerging evidence shows that the extensive cross-interactions revealed by tissue culture analysis of the EGF system has profound importance in vivo. This is manifested by the striking phenotypic similarities of mice with homozygous disruptions of the ErbB2, ErbB4, and NRG genes.[5-8] These three gene disruptions are all embryonically lethal at day 10.5 postcoitum and induce overlapping but not identical defects in the nervous system. Significantly, these animals all lack the trabecular extensions of the ventricular myocardium, which results in lethality due to cardiac malfunction. ErbB2 and ErbB4 are expressed in the myocardium, whereas NRG is expressed in the endocardium. The disruption phenotypes show that paracrine activation of both ErbB2 and ErbB4 by NRG is required for proper myocardial development and verifies the importance of transmodulation of ErbB2 in a physiological response to NRG.[4,62]

A number of open questions regarding the regulation and coupling of this signaling network remain. For the hormones, there is little understanding of the role of the non-EGF homologous domains, which can be quite large and contain a number of recognizable motifs. For example, some of the differentially spliced NRG isoforms include an immunoglobulin (Ig)-like domain, a glycosylation-rich spacer domain, a cysteine-rich "sensory and motor neuron-derived factor" domain, and a variant kringle domain.[9,63] Although these domains are not required for activation of ErbB3 or ErbB4, they may regulate hormone-receptor interactions in a quantitative manner. In fact, mutant mice homozygous for a mutant NRG gene containing a disruption in the Ig-like domain die during embryogenesis and exhibit neurologic and cardiac defects similar to those observed in mice homozygous for complete disruption of the NRG gene.[5,8] Although this implies that the Ig-like domain is required for ErbB family receptor activation, another possibility is that this domain is required for NRG stabilization or presentation by extracellular matrix in vivo. In a similar vein, only a little more is known about regulation of hormone-receptor interactions by heparin-sulfate proteoglycans (HSPGs). NRG, HB-EGF, and AR bind HSPGs, and HSPGs regulate the interactions of HB-EGF and AR with the EGFR.[64] HSPGs may regulate ligand binding by acting as low-affinity receptors for AR and HB-EGF, increasing the local concentration of these hormones.

EGF family hormones are initially synthesized as membrane-anchored precursors that are subsequently cleaved to release soluble hormone. Experiments using mice homozygous for disruptions in the ErbB4 and NRG genes suggest that activation of ErbB family receptors by membrane-anchored hormones on an adjacent cell or tissue (juxtacrine regulation) is required for some developmental processes. It is unclear, however, whether the membrane-anchored and soluble forms of EGF family hormones stimulate identical patterns of receptor tyrosine phosphorylation. Steric and conformational constraints on the membrane-anchored hormones might restrict their activities. Several of the membrane-anchored hormone precursors possess significant cytoplasmic tails. During juxtracrine receptor activation the membrane-anchored hormones also may act as receptors, coupling to signaling effectors and physiological changes in response to ErbB family receptor binding. This possibility has stimulated efforts to identify proteins that interact with the cytoplasmic domains of EGF family prohormones. The cytoplasmic domain of TGF-α has been shown to associate with an uncharacterized protein kinase activity as well as a p86 cytoplasmic protein. However, the identities of the protein kinase and the p86 cytoplasmic protein have yet to be determined, and the regulation of these interactions has yet to be defined.[65,66]

Another emerging field of study is the role of ErbB family receptors as effectors for stimuli independent of EGF family hormones,[67] much as nonreceptor protein tyrosine kinases serve as signaling effectors for receptors lacking kinase activity. Treatment of HeLa cells with short-wavelength ultraviolet light (UVC) stimulates EGFR and p42 MAP kinase phosphorylation and induces c-*fos* and c-*jun* transcription. These responses are not seen in HeLa cells ectopically expressing a dominant-negative EGFR mutant.[68] It has been proposed that one mechanism by which UV induces cellular responses is through the induction of reactive oxygen species (ROS), such as hydrogen peroxide. Therefore, it is not surprising that in vascular smooth muscle cells hydrogen peroxide also stimulates the phosphorylation of EGFR and MAP kinases as well as the formation of complexes containing Shc, Grb2, SOS, and EGFR.[69] Surprisingly, EGF stimulates hydrogen peroxide generation in A431 human carcinoma cells, and eliminating hydrogen peroxide with catalase reduces EGFR tyrosine phosphorylation in response to EGF. It has been proposed that hydrogen peroxide inhibits a protein phosphatase specific for the EGFR and that this

inhibition is required for the maintenance of EGFR in its phosphorylated state.[70] Therefore, cellular stress, which in some cases is accompanied by ROS release, may be coupled to the MAP kinase signaling pathway through EGFR and perhaps other ErbB family receptors as well.

There is also increasing evidence that G protein-coupled serpentine receptors (GPCR) also regulate ErbB family receptor signaling. The GPCR ligands thrombin, endothelin-1, and lysophosphatidic acid all activate Neu, EGFR, Shc, Grb2, and the ERK1 and ERK2 MAP kinases in Rat-1 fibroblasts. Furthermore, activation of these signaling pathways by GPCR ligands is disrupted in Rat-1 cells expressing a dominant negative EGFR mutant or in Rat-1 cells preincubated with the EGFR antagonist tyrphostin AG1478.[71] The mechanism for ErbB family receptor phosphorylation in response to GPCR ligands is still unclear, although it has been proposed that src family kinases may be involved. An interesting twist is that the purified EGFR phosphorylates the $G_{s\alpha}$ subunit of the heterotrimeric G protein complex in vitro and that phosphorylated $G_{s\alpha}$ displays increased GTPase activity and greater GTPγS binding capacity and that phospho-$G_{s\alpha}$ augmented adenyl cyclase activity in S49 cyc⁻ cell membranes.[72,73] Because EGF stimulates cAMP accumulation in the heart via $G_{s\alpha}$,[72] cross-talk between ErbB family receptors and the GPCR signal transduction pathway may be bidirectional.

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REFERENCES

1 Hynes, N.E., and Stern, D.F. (1994). The biology of erbB2/neu/HER2 and its role in cancer. *Biochim Biophys Acta* **1198,** 165–184.

2 Salomon, D.S., Brandt, R., Ciardiello, F., and Normanno, N. (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19,** 183–232.

3 Fischbach, G.D., and Rosen, K.M. (1997). ARIA: A neuromuscular junction neuregulin. *Annu Rev Neurosci* **20,** 429–458.

4 Carraway, K.L. III (1996). Involvement of the neuregulins and their receptors in cardiac and neural development. *Bioessays* **18,** 263–266.

5 Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378,** 386–390.

6 Gassmann, M., Casagranda, F., Orioll, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378,** 390–394.

7 Lee, K.-F., Simon, H., Chen, H., Bates, B., Hung, M.-C., and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378,** 394–398.

8 Kramer, R., Bucay, N., Kane, D.J., Martin, L.E., Tarpley, J.E., and Theill, L.E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc Natl Acad Sci USA* **93,** 4833–4838.

9 Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Berminghan-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., McBurney, R.N., Waterfield, M.D., Stroobant, P., and Gwynne, D. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature **362,** 312–318.

10 Chang, H., Riese, D.J. II, Gilbert, W., Stern, D.F., and McMahan, U.J. (1997). Ligands for ErbB family receptors encoded by a newly-characterized neuregulin-like gene. *Nature* **387,** 509–512.

11 Carraway, K.L. III, Weber, J.L., Under, M.J., Ledesman, J., Yu, N., Gassmann, M., and Lai, C. (1997). Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature* **387,** 512–516.

12 Ciccodicola, A., Dono, R., Obici, S., Simeone, A., Zollo, M., and Persico, M.G. (1989). Molecular characterization of a gene of the "EGF family" expressed in undifferentiated human NTERA2 teratocarcinoma cells. *EMBO J* **8,** 1987–1991.

13 Brandt, R., Normanno, N., Gullick, W.J., Lin, J.H., Harkins, R., Schneider, D., Jones, B.W., Ciardiello, F., Persico, M.G., Armenante, F., Kim, N., and Salomon, D.S. (1994). Identification and biological characterization of an epidermal growth factor-related protein: Cripto-1. *J Biol Chem* **269,** 17320–17328.

14 Shen, M.M., Wang, H., and Leder, P. (1997). A differential display strategy identifies cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. *Development* **124,** 429–442.

15 Kannan, S., DeSantis, M., Lohmeyer, M., Riese, D.J. II, Smith, G.H., Hynes, N., Seno, M., Brandt, R., Bianco, C., Persico, G., Kenney, N., Normanno, N., Martinez-Lacaci, I., Ciardiello, F., Stern, D.F., Gullick, W.J., and Salomon, D.S . (1997). Cripto enhances the tyrosine phosphorylation of Shc and activates MAP-Kinase in mammary epithelial cells. *J Biol Chem* **272,** 3330–3335.

16 Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Liebermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P.H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309,** 418–425.

17 Schechter, A.L., Hung, M.C., Vaidyanathan, L., Weinberg, R.A., Yang-Feng, T.L, Francke, U., Ullrich, A., and Coussens, L. (1985). The neu gene: An erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* **229,** 976–978.

18 Semba, K., Kamata, N., Toyoshima, K., and Yamamoto, T. (1985). A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary adenocarcinoma. *Proc Natl Acad Sci USA* **82,** 6497–6501.

19 Kraus, M.H., Issing, W., Miki, T., Popescu, N.C., and Aaronson, S.A. (1989). Isolation and characterization of ErbB3, a third member of the ErbB/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci USA* **86,** 9193–9197.

20 Plowman, G.D., Whitney, G.S., Neubauer, M.G., Green, J.M., McDonald, V.L., Todaro, G.J., and Shoyab, M. (1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci USA* **87,** 4905–4909.

21 Plowman, G.D., Culouscou, J.-M., Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G., and Shoyab, M. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci USA* **90,** 1746–1750.

22 Stern, D.F., Heffernan, P.A., and Weinberg, R.A. (1986). p185, a product of the neu proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. *Mol Cell Biol* **6,** 1729–1740.

23 Stern, D.F., and Kamps, M.P. (1988). EGF-stimulated tyrosine phosphorylation of $p185^{neu}$: A potential model for receptor interactions. *EMBO J* **7,** 995–1001.

24 King, C.R., Borrello, I., Bellot, F., Comoglio, P., and Schlessinger, J. (1988). EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3. *EMBO J* **7,** 1647–1651.

25 Connelly, P.A., and Stern, D.F. (1990). The epidermal growth factor receptor and the product of the neu protooncogene are members of a receptor tyrosine kinase cascade. *Proc Natl Acad Sci USA* **87,** 6054–6057.

26 Goldman, R., Ben Levy, R., Peles, E., and Yarden, Y. (1990). Heterodimerization of the ErbB1 and ErbB2 receptors in human breast carcinoma cells: A mechanism for receptor transregulation. *Biochemistry* **29,** 11024–11028.

27 Wada, T., Qian, X., and Greene, M.I. (1990). Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function. *Cell* **61,** 1339–1347.

28 Muthuswamy, S.K., and Muller, W.J. (1995). Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. *Oncogene* **11,** 271–279.

29 Riese, D.J. II, Bermingham, Y., van Raaij, T.M., Buckley, S., Plowman, G.D., and Stern, D.F. (1996). Betacellulin activates the epidermal growth factor receptor and ErbB4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β. *Oncogene* **12,** 345–353.

30 Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B., and Yarden, Y. (1996). Neu differentiation factor/Neuregulin isoforms activate distinct receptor combinations. *J Biol Chem* **271,** 19029–19032.

31 Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B.J., Sela, M., and Yarden, Y. (1996). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* **15,** 2452–2467.

32 Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B.J., and Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* **16,** 5276–5287.

33 Graus-Porta, D., Beerli, R.R., Daly, J.M., and Hynes, N.E. (1997). ErbB2, the preferred heterodimerization partner of all erbB receptors, is a mediator of lateral signaling. *EMBO J* **16,** 1647–1655.

34 Karunagaran, D., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1995). Neu differentiation factor inhibits EGF binding. *J Biol Chem* **270,** 9982–9990.

35 Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L., and Carraway, K.L. III. (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J Biol Chem* **269,** 14661–14665.

36 Karunagaran, D., Tzahar, E., Beerli, R., Chen, X., Graus-Porta, D., Ratzkin, B.J., Seger, R., Hynes, N.E., and Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: Implications for breast cancer. *EMBO J* **15,** 254–264.

37 Sherill, J.M., and Kyte, J. (1996). Activation of epidermal growth factor receptor by epidermal growth factor. *Biochemistry* **35,** 5705–5718.

38 Lemmon, M.A., Bu, Z., Ladbury, J.E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D.M., and Schlessinger, J. (1997). Two EGF molecules contribute additively to stabilization of the EGFR dimer. *EMBO J* **16,** 281–294.

39 Barbacci, E.G., Guarino, B.C., Stroh, J.G., Singleton, D.H., Rosnack, K.J., Moyer, J.D., and Andrews, G.C. (1995). The structural basis for the specificity of epidermal growth factor and heregulin binding. *J Biol Chem* **270,** 9585–9589.

40 Gamett, D.C., Pearson, G., Cerione, R.A., and Friedberg, I. (1997). Secondary dimerization between members of the epidermal growth factor receptor family. *J Biol Chem* **272,** 12052–12056.

41 Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A., and Carraway, K.L. III. (1994). Insect cell-expressed $p180^{erbB3}$ possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci USA* **91,** 8132–8136.

42 Carraway, K.L. III, Sliwkowski, M.X., Akita, R., Platko, J.V., Guy, P.M., Nuijens, A., Diamonti, A.J., Vandlen, R.L., Cantley, L.C., and Cerione, R.A. (1994). The erbB3 gene product is a receptor for heregulin. *J Biol Chem* **269,** 14303–14306.

43 Riese, D.J. II, van Raaij, T.M., Plowman, G.D, Andrews, G.C., and Stern, D.F. (1995). The cellular response to neuregulins is governed by complex interactions of the ErbB receptor family. *Mol Cell Biol* **15,** 5770–5776.

44 Hudziak, R.M., Schlessinger, J., and Ullrich, A. (1987). Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH3T3 cells. *Proc Natl Acad Sci USA* **84,** 7159–7163.

45 DiFiore, P.P., Pierce, J.H., Kraus, M.H., Segatto, O., King, C.R., and Aaronson, S.A. (1987b). ErbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* **237,** 179–182.

46 DiFiore, P.P., Segatto, O., Lonardo, F., Fazioli, F., Pierce, J.H., and Aaronson, S.A. (1990). The carboxyl-terminal domains of erbB-2 and epidermal growth factor receptor exert different regulatory effects on intrinsic receptor tyrosine kinase function and transforming activity. *Mol Cell Biol* **10,** 2749–2756.

47 Segatto, O., Lonardo, F., Wexler, D., Fazioli, F., Pierce, J.H., Bottaro, D.P., White, M.F., and DiFiore, P.P. (1991). The juxtamembrane regions of the epidermal growth factor receptor and $gp185^{erbB2}$ determine the specificity of signal transduction. *Mol Cell Biol* **11,** 3191–3202.

48 Alimandi, M., Romano, A., Curia, M.C., Muraro, R., Fedi, P., Aaronson, S.A., DiFiore, P.P., and Kraus, M.H. (1995). Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene* **10,** 1813–1821.

49 Zhang, K., Sun, J., Liu, N., Wen, D., Chang, D., Thomason, A., and Yoshinaga, S.K. (1996). Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. *J Biol Chem* **271,** 3884–3890.

50 Cohen, B.D., Green, J.M., Foy, L., and Fell, H.P. (1996). HER4-mediated biological and biochemical properties in NIH3T3 cells. *J Biol Chem* **271,** 4813–4818.

51 Cohen, B.D., Kiener, P.A., Green, J.M., Foy, L., Fell, H.P., and Zhang, K. (1996). The relationship between human epidermal growth-like factor receptor expression and cellular transformation in NIH3T3 cells. *J Biol Chem* **271,** 30897–30903.

52 Fazioli, F., Kim, U.-H., Rhee, S.G., Molloy, C.J., Segatto, O., and DiFiore, P.P. (1991). The erbB2 mitogenic signaling pathway: Tyrosine phosphorylation of phospholipase C-γ and GTPase activating protein does not correlate with erbB2 mitogenic potency. *Mol Cell Biol* **11,** 2040–2048.

53 Kokai, Y., Myers, J.N., Wada, T., Brown, V.I., LeVea, C.M., Davis, J.G., Dobashi, K., and Greene, M.I. (1989). Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. *Cell* **58,** 287–292.

54 Carter, T.H., and Kung, H.J. (1994). Tissue specific transformation by oncogenic mutants of epidermal growth factor receptor. *Crit Rev Oncog* **5,** 389–428.

55 van der Geer, P., Hunter, T., and Lindberg, R.A. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol* **10,** 521–337.

56 Kumar, V., Bustin, S.A., and McKay, I.A. (1995). Transforming growth factor alpha. *Cell Biol Int* **19,** 373–388.

57 Hunter, T. (1995). Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80,** 225–236.

58 Earp, H.S., Dawson, T.L., Li, X., and Yu, H. (1995) Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* **35,** 115–132.

59 Bacus, S.S., Yarden, Y., Oren, M., Chin, D.M., Lyass, L., Zelnick, C.R., Kazarov, A., Toyofuku, W., Gray-Bablin, J., Beerli, R., Hynes, N.E., Nikiforov, M., Haffner, R., Gudkov, A., and Keyomarsi, K. (1996). Neu differentiation factor (Heregulin) activates a p53-dependent pathway in cancer cells. *Oncogene* **12,** 2535–2547.

60 Levkowitz, G., Klapper, L.N., Tzahar, E., Freywald, A., Sela, M., and Yarden, Y. (1996). Coupling of the c-Cbl protooncogene product to ErbB1/EGF receptor but not to other ErbB proteins. *Oncogene* **12,** 1117–1125.

61 Zrihan-Licht, S., Lim, J., Keydar, I., Sliwkowski, M.X., Groopman, J.E., and Avraham, H. (1997). Association of Csk-homologous Kinase (CHK) (formerly MATK) with HER-2/ErbB-2 in breast cancer cells. *J Biol Chem* **272,** 1856–1863.

62 Marchionni, M.A. (1995). Neu tack on neuregulin. *Nature* **378,** 334–335.

63 Lemke, G. (1996). Neuregulins in development. *Mol Cell Neurosci* **7,** 247–262.

64 Cook, P.W., Ashton, N.M., Karkaria, C.E., Siess, D.C., and Shipley, G.D. (1995). Differential effects of a heparin antagonist (hexadimethrine) or chlorate on amphiregulin, basic fibroblast growth factor, and heparin-binding EGF-like growth factor activity. *J Cell Physiol* **163,** 418–429.

65 Shum, L., Reeves, S.A., Kuo, A.C., Fromer, E.S., and Derynck R. (1994). Association of the transmembrane TGF-alpha precursor with a protein kinase complex. *J Cell Biol* **125,** 903–916.

66 Shum, L., Turck, C.W., and Derynck, R. (1996). Cysteines 153 and 154 of transmembrane transforming growth factor-alpha are palmitoylated and mediate cytoplasmic protein association. *J Biol Chem* **271,** 28502–28508.

67 Weiss, F.U., Daub, H., and Ullrich, A. (1997). Novel mechanisms of RTK signal generation. *Curr Opin Genet Dev* **7,** 80–86.

68 Sachsenmaier, C., Radler-Pohl, A., Zinck, R., Nordheim, A., Herrlich, P., and Rahmsdorf, H.J. (1994). Involvement of growth factor receptors in the mammalian UVC response. *Cell* **78,** 963–972.

69 Rao, G.N. (1996). Hydrogen peroxide induces complex formation of Shc-Grb2-SOS with receptor tyrosine kinase and activates Ras and extracellular signal-regulated protein kinases group of mitogen-activated protein kinases. *Oncogene* **13,** 713–719.

70 Bae, Y.S., Kang, S.W., Seo, M.S., Baines, I.C., Tekle, E., Chock, P.B., and Rhee, S.G. (1997). Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. *J Biol Chem* **272,** 217–221.

71 Daub, H., Weiss, F.U., Wallasch, C., and Ullrich, A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379,** 557–560.

72 Nair, B.G., Parikh, B., Milligan, G., and Patel, T.B. (1990). $G_{s\alpha}$ mediates epidermal growth factor-elicited stimulation of rat cardiac adenylate cyclase. *J Biol Chem* **265,** 21317–23122.

73 Poppleton, H., Sun, H., Fulgham, D., Bertics, P., and Patel, T.B. (1996). Activation of $G_{s\alpha}$ by the epidermal growth factor receptor involves phosphorylation. *J Biol Chem* **271,** 6947–6951.

74 Shoyab, M., Plowman, G.D., McDonald, V.L., Bradley, J.G., and Todaro, G.J. (1989). Structure and function of human amphiregulin: A member of the epidermal growth factor family. *Science* **243,** 1074–1076.

75 Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C., and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251,** 936–939.

76 Johnson, G.R., Kannan, B., Shoyab, M., and Stromberg, K. (1993). Amphiregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and $p185^{erbB2}$. *J Biol Chem* **268,** 2924–2931.

77 Plowman, G.D., Green, J.M., Culouscou, J.-M., Carlton, G.W., Rothwell, V.M., and Buckley, S. (1993). Heregulin induces tyrosine phosphorylation of $Her4/p180^{erbB4}$. *Nature* **366,** 473–475.

78 Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K., and Folkman, J. (1993). Betacellulin: A mitogen from pancreatic β cell tumors. *Science* **259,** 1604–1607.

79 Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K. (1995). Epiregulin: A novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *J Biol Chem* **270,** 7495–7500.

80 Riese, D.J. II, Kim, E.D., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G.D., and Stern, D.F. (1996). The epidermal growth factor receptor couples transforming growth factor-α, heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB3, and ErbB4. *J Biol Chem* **271,** 20047–20052.

81 Beerli, R.R., and Hynes, N.E. (1996). Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities. *J Biol Chem* **271,** 6071–6076.

82 Elenius, K., Paul, S., Allison, G., Sun, J., and Klagsbrun, M. (1997). Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J* **16,** 1268–1278.

83 Riese, D.J. II, Komurasaki, T., Plowman, G.D., and Stern, D.F. Activation of ErbB4 by the bifunctional EGF family hormone epiregulin is regulated by ErbB2. (submitted).

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Activation of ErbB4 by the Bifunctional Epidermal Growth Factor Family Hormone Epiregulin Is Regulated by ErbB2*

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Epiregulin (EPR) is a recently described member of the epidermal growth factor (EGF) family of peptide growth factors. The ever expanding size of the EGF family has made distinguishing the activities of these hormones paramount. We show here that EPR activates two members of the ErbB family of receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ErbB4. Therefore by these criteria, EPR is qualitatively similar to another EGF family hormone, betacellulin (BTC). Yet, here we also demonstrate quantitative differences between EPR and BTC. EPR stimulates higher levels of EGFR phosphorylation than does BTC, whereas BTC stimulates higher levels of ErbB4 phosphorylation than does EPR. Moreover, the EPR and BTC dose response curves show that although EGFR is more sensitive to EPR than is ErbB4, ErbB4 is more sensitive to BTC than is EGFR. Finally, ErbB2, which is not activated by EPR when expressed on its own, increases the sensitivity of ErbB4 for activation by EPR. Therefore, these results establish that EPR exhibits novel activities and modes of regulation, which may have significant implications for EPR function *in vivo*.

The continuing discovery of novel members of the epidermal growth factor (EGF)[1] family of peptide growth factors has led to an increased appreciation of the functional differences among these hormones, as well as a realization of the complex hormone-receptor interactions fostered by these peptides. EGF, transforming growth factor α (TGF-α), and amphiregulin all bind exclusively to the EGF receptor (EGFR). Yet, these hormones can also activate *in trans* (transmodulate) the other three ErbB family receptors (Neu/ErbB2/Her2, ErbB3/Her3, ErbB4/Her4) through ligand-induced receptor heterodimerization with the EGFR (1–8). Other EGF family hormones bind multiple receptors. Neuregulin (NRG) and neuregulin2 (NRG2) bind ErbB3 and ErbB4 and transmodulate EGFR and ErbB2 (9–16). Betacellulin (BTC) combines some of the properties of EGF and NRG by activating EGFR and ErbB4 (7).

EPR was initially purified from the conditioned medium of a tumorigenic clone of NIH3T3 fibroblasts. It competes with EGF for binding to A431 cells, which overexpress EGFR, suggesting that EPR is a ligand for EGFR (17). Since at least one of the EGF family hormones that activates EGFR also activates ErbB4, we wished to evaluate EPR function in a set of cell lines expressing all four ErbB family receptors, both singly and in every pairwise combination.

We demonstrate here that EPR activates not only EGFR, but ErbB4 as well. However, the dose-response curves for BTC and EPR in a cell line expressing both EGFR and ErbB4 are markedly different. Whereas ErbB4 is more responsive to BTC than is EGFR, ErbB4 is less responsive to EPR than is EGFR. Moreover, ErbB2 expression increases saturated ErbB4 phosphorylation in response to EPR and dramatically enhances the sensitivity of ErbB4 for activation by EPR as well. In this respect EPR resembles NRG, which displays a low affinity for ErbB3 that increases in cells where ErbB2 is co-expressed (12).

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—BaF3 is an immortal mouse lymphoblastoid cell line (31). BaF3-derived cell lines expressing combinations of ErbB family receptors have been described previously (14). The ranked order of receptor expression in the double recombinant BaF3 cell lines is as follows. For EGFR expression, BaF3/EGFR+ErbB4 is higher than BaF3/EGFR+ErbB2, which is higher than BaF3/EGFR+ErbB3. For ErbB2 expression, BaF3/ErbB2+ErbB4 is equivalent to BaF3/EGFR+ErbB2, both of which are markedly higher than BaF3/ErbB2+ErbB3. The levels of ErbB3 expression are similiar in the BaF3/EGFR+ErbB3, BaF3/ErbB2+ErbB3, and BaF3/ErbB3+ErbB4 cell lines. The levels of ErbB4 expression are similiar in the BaF3/EGFR+ErbB4, BaF3/Neu+ErbB4, and BaF3/ErbB3+ErbB4 cell lines (7, 14).

CEM is an immortal human T-lymphoblastoid cell line that does not endogenously express EGF receptor, ErbB2, ErbB3, or ErbB4. CEM-derived cell lines expressing ErbB4 or ErbB2 and ErbB4 have been described previously (10). Cell culture conditions were as described (10, 14).

Growth Factors—Recombinant human EPR was produced in *Bacillus brevis*.[2] Recombinant NRGβ was the generous gift of Kerry Russell and Jeffrey Bender (Yale University). We are grateful to Jim Moyer, Brad Guarino, and Glenn Andrews (Pfizer Central Research, Groton, CT) for synthetic NRGβ (32). Recombinant BTC and NRGβ were purchased from R & D Systems (Minneapolis, MN), whereas recom-

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[1] The abbreviations used are: EGF, epidermal growth factor; TGF-α, transforming growth factor α; EGFR, EGF receptor; NRG, neuregulin; BTC, betacellulin; IL3, interleukin 3; PAGE, polyacrylamide gel electrophoresis.

[2] T. Nakazawa *et al.*, in preparation.

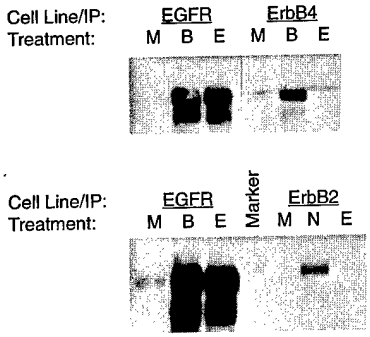

FIG. 1. **EPR stimulation of receptor phosphorylation in Ba/F3 cells expressing a single ErbB family receptor.** BaF3/EGFR, BaF3/ErbB2, and BaF3/ErbB4 cells (14) were stimulated with 1000 ng/ml EPR (*E*), 100 ng/ml BTC (*B*), 100 ng/ml recombinant NRGβ (*N*), or were mock stimulated with phosphate-buffered saline (*M*) as described previously (7, 14). ErbB family receptors were immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

binant TGF-α was purchased from Collaborative Biomedical Products (Bedford, MA).

Stimulation and Analysis of Receptor Phosphorylation—The conditions for stimulation of ErbB family receptor tyrosine phosphorylation have been described previously (7, 14). The analysis of ErbB family receptor tyrosine phosphorylation by immunoprecipitation and antiphosphotyrosine immunoblotting has been described previously (7, 14). Immunoprecipitating antireceptor antibodies were anti-EGFR mouse monoclonal antibody 528 (33), anti-ErbB2 mouse monoclonal antibody TA-1 (OP-39, Calbiochem), anti-ErbB3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology), and anti-ErbB4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of antireceptor antibodies has been verified by testing for cross-reactivity (data not shown).

Immunoblot autoradiographs were digitized using a Hewlett-Packard 3p flatbed scanner set for 600 dpi resolution and controlled by Hewlett-Packard Deskscan II for Macintosh software. Images were cropped using Adobe Photoshop, and the band intensity was quantified using NIH Image software. Net receptor activation was calculated by subtracting the amount of tyrosine phosphorylation observed in samples from mock-stimulated cells.

RESULTS

EPR Activates EGFR—We first sought to identify which ErbB family receptors are activated by EPR when the receptors are expressed individually. We previously developed a panel of cell lines based on the mouse BaF3 hematopoietic cell line that expresses the four ErbB family receptors, both singly and in every pairwise combination. Hence, we incubated the BaF3 cell lines ectopically expressing EGFR, ErbB2, or ErbB4 with EPR. EPR, like BTC, stimulated EGFR tyrosine phosphorylation, consistent with published results suggesting that EPR binds EGFR (17) (Fig. 1, *EGFR panel*; compare *lanes E* and *B* with *M*). However, EPR did not stimulate phosphorylation of ErbB2 or ErbB4 (Fig. 1, *ErbB2* and *ErbB4 panels*; compare *lanes E* and *M*). In contrast, the positive control NRGβ stimulated ErbB2 tyrosine phosphorylation and BTC stimulated ErbB4 phosphorylation. The ErbB2 phosphorylation observed in BaF3/ErbB2 cells stimulated with NRG is the result of transmodulation of ErbB2 by the NRG receptor ErbB3, which is endogenously expressed at low levels in BaF3 cells (14). Neither EPR nor any of the other EGF family ligands tested to date stimulated ErbB3 tyrosine phosphorylation in BaF3 cells expressing only ErbB3 (data not shown) (7, 8, 14). However, since ErbB3 lacks tyrosine kinase activity (18), these experiments do not rule out EPR binding to ErbB3.

Since EPR activates EGFR, we next determined whether EPR activates the other three ErbB family receptors *in trans*

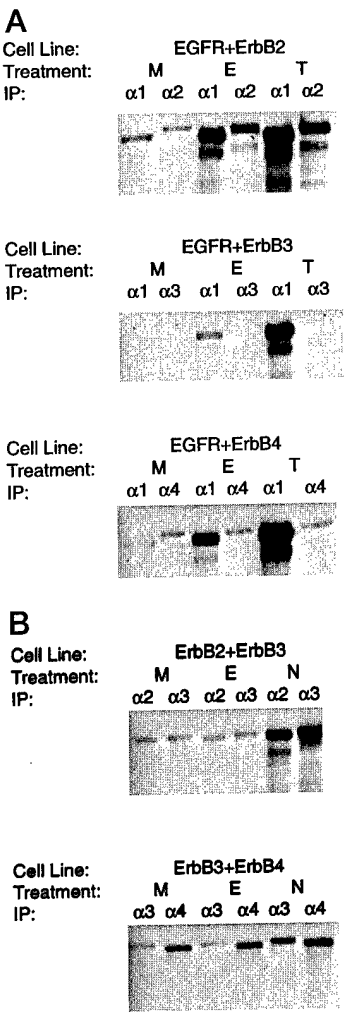

FIG. 2. ***A* and *B*, EPR stimulation of receptor phosphorylation in BaF3 cells expressing combinations of ErbB family receptors.** BaF3/EGFR+ErbB2, BaF3/EGFR+ErbB3, BaF3/EGFR+ErbB4, BaF3/ErbB2+ErbB3, and BaF3/ErbB3+ErbB4 cells (14) were stimulated with 200 ng/ml EPR (*E*), 200 ng/ml TGF-α (*T*), 188 ng/ml synthetic NRGβ (*N*), or were mock stimulated with phosphate-buffered saline (*M*) as described previously (7, 8, 14). EGFR (α*1*), ErbB2 (α*2*), ErbB3 (α*3*), or ErbB4 (α*4*) was immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

via EGFR. A panel of BaF3 cell lines ectopically expressing EGFR together with one of the other three ErbB family receptors was stimulated with EPR. EPR activated the EGFR in all three cell lines (Fig. 2*A*; compare *E* α*1 lanes* with the *M* α*1 lanes*). Both EPR (*E lanes*) and the positive control TGF-α (*T lanes*) strongly activated ErbB2 in the cell line co-expressing EGFR+ErbB2 (Fig. 2*A*, *EGFR+ErbB2 panel*; α*2 lanes*). In contrast, neither EPR nor TGF-α activated ErbB3 or ErbB4 (Fig. 2*A*, *EGFR+ErbB3* and *EGFR+ErbB4 panels*; α*3* or α*4 lanes*). This is consistent with the conclusion that ErbB2 is a preferential target for transmodulation by the EGFR compared with the other ErbB family receptors (7, 8, 19–21). However, higher concentrations of EPR than those used for these experiments did stimulate ErbB4 phosphorylation in the EGFR+ErbB4 cell line (see below; Fig. 6*A*).

Since ErbB3 lacks functional kinase activity, EGF family hormones can activate ErbB3 only in the presence of another ErbB family receptor, particularly ErbB2, which permits the highest levels of ErbB3 phosphorylation by NRG (11, 12, 14).

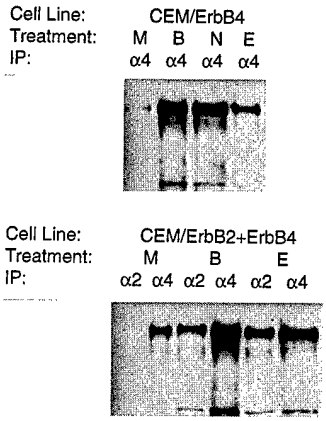

FIG. 3. **EPR stimulation of receptor phosphorylation in CEM cells expressing either ErbB4 alone or both ErbB2 and ErbB4.** CEM/ErbB4 and CEM/ErbB2+ErbB4 cells (10) were stimulated with 100 ng/ml BTC (*B*), 100 ng/ml recombinant NRGβ (*N*), 1000 ng/ml EPR (*E*), or were mock stimulated with phosphate-buffered saline (*M*) as described previously (14). ErbB2 ($\alpha 2$) or ErbB4 ($\alpha 4$) was immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

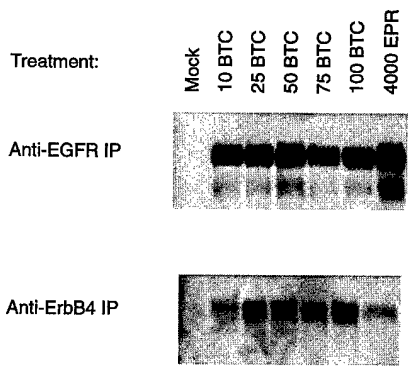

FIG. 4. **Stimulation of receptor phosphorylation in BaF3 cells expressing both EGFR and ErbB4 by EPR and increasing doses of BTC.** BaF3/EGFR+ErbB4 cells (14) were stimulated with 10, 25, 50, 75, or 100 ng/ml BTC, 4000 ng/ml EPR, or mock stimulated with phosphate-buffered saline as described previously (7, 8, 14). EGFR or ErbB4 was immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

Therefore, BaF3 cells expressing both ErbB2 and ErbB3 or both ErbB3 and ErbB4 were stimulated with EPR to determine if ErbB3 is a receptor for EPR. In the ErbB2+ErbB3 cell line, the positive control NRGβ activated both receptors (Fig. 2*B*, *ErbB2+ErbB3 panel*, compare *N lanes* with *M lanes*), whereas in the ErbB3+ErbB4 cell line NRGβ stimulated a marked increase in ErbB3 phosphorylation and a modest increase in ErbB4 phosphorylation (Fig. 2*B*, *ErbB3+ErbB4* panel, compare *N lanes* with *M lanes*). In contrast, EPR did not stimulate receptor phosphorylation in either of these cell lines, suggesting that ErbB3 is not a receptor for EPR (Fig. 2*B*; compare *E lanes* with *M lanes*).

EPR Activates ErbB4 in CEM Cells—BTC activates both ErbB4 and EGFR when expressed individually (7). We tested whether EPR behaves like BTC and also activates ErbB4 when expressed alone using derivatives of the CEM human T-lymphoblastoid cell line that ectopically expresses ErbB4 alone or both ErbB2 and ErbB4 (10). EPR activated ErbB4 in CEM cells expressing ErbB4 alone and both receptors in CEM cells expressing ErbB2 and ErbB4 together (Fig. 3; compare *E lanes* with *M lanes*). In experiments done in parallel using identical growth factor concentrations, EPR did not activate ErbB4 in BaF3 cells expressing ErbB4 alone (also see Fig. 1) but stimulated ErbB2 and ErbB4 phosphorylation in BaF3 cells expressing both ErbB2 and ErbB4 (data not shown). It is unclear why EPR failed to activate ErbB4 in the BaF3 cells expressing ErbB4 alone. Nonetheless, because EPR activates EGFR as well as ErbB4, EPR resembles BTC, which also activates these receptors (7).

EPR Stimulates EGFR More Than ErbB4 and EGFR Is More Sensitive Than ErbB4 to EPR—Since BTC and EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we measured BTC and EPR stimulation of EGFR and ErbB4 phosphorylation in BaF3 cells that express both receptors together (Fig. 4). BTC stimulated saturated levels of EGFR phosphorylation at a concentration of 10 ng/ml, whereas BTC stimulated saturated levels of ErbB4 phosphorylation at a concentration of 25 ng/ml. Therefore, in subsequent experiments we have assumed that 100 ng/ml BTC stimulates saturated levels of EGFR and ErbB4 phosphorylation.

At a concentration of 4000 ng/ml, EPR stimulates slightly greater levels of EGFR phosphorylation than does BTC (Fig. 4). In contrast, 4000 ng/ml EPR stimulates much lower levels of ErbB4 phosphorylation than does BTC (Fig. 4). This suggests that EPR stimulates EGFR more than ErbB4 and that EGFR is more sensitive than ErbB4 to EPR. We investigated this possibility by comparing EGFR and ErbB4 phosphorylation in BaF3/EGFR and CEM/ErbB4 cells stimulated with increasing concentrations of EPR (Fig. 5*A*). At saturation, EPR stimulated a slightly higher level of EGFR phosphorylation (1.7-fold) than did BTC, whereas EPR stimulated a much lower level of ErbB4 phosphorylation (0.3-fold) than did BTC (Fig. 5*C*). We next compared the dose sensitivity of EGFR and ErbB4 to EPR stimulation by plotting receptor phosphorylation relative to the maximal amounts of receptor phosphorylation stimulated by EPR (Fig. 5*D*) to identify the EPR concentrations required for half-maximal receptor phosphorylation (Table I). Half-maximal EGFR phosphorylation occurred at an EPR concentration of approximately 380 ng/ml (Fig. 5*D*; Table I). In contrast, half-maximal ErbB4 phosphorylation required about a 4-fold higher concentration of EPR with half-maximal activation occurring at an EPR concentration of approximately 1790 ng/ml (Fig. 5*D*; Table I).

ErbB2 Expression Increases ErbB4 Activation by EPR and Sensitivity to EPR—The affinity of NRG for cells expressing ErbB3 is greater when these cells also express ErbB2 (12). Hence, we evaluated the possibility that ErbB2 modulates ErbB4 activation by EPR by stimulating CEM/ErbB4 and CEM/ErbB2+ErbB4 cells with increasing concentrations of EPR (Fig. 5, *A* and *B*). Relative to the BTC positive control, EPR stimulated 2-fold higher levels of ErbB4 phosphorylation in the ErbB2+ErbB4 cell line than in cells expressing ErbB4 alone (Fig. 5*C*). Therefore, ErbB2, which is not activated by EPR when expressed by itself (Fig. 1), doubles the magnitude of ErbB4 activation by EPR at saturation. We next examined the effects of ErbB2 expression on the sensitivity of ErbB4 to EPR (Fig. 5*D*). Half-maximal ErbB4 phosphorylation occurred at an EPR concentration of approximately 1790 ng/ml in the cell line expressing ErbB4 alone (Fig. 5*D*; Table I), but occurred at an EPR concentration of approximately 630 ng/ml (Fig. 5*D*; Table I) in the ErbB2+ErbB4 cell line. This shift in the EPR dose-response curve in the ErbB2+ErbB4 cell line compared with the ErbB4 cell line suggests that ErbB2 expression increases the affinity of ErbB4 for EPR and implies that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than do ErbB4-

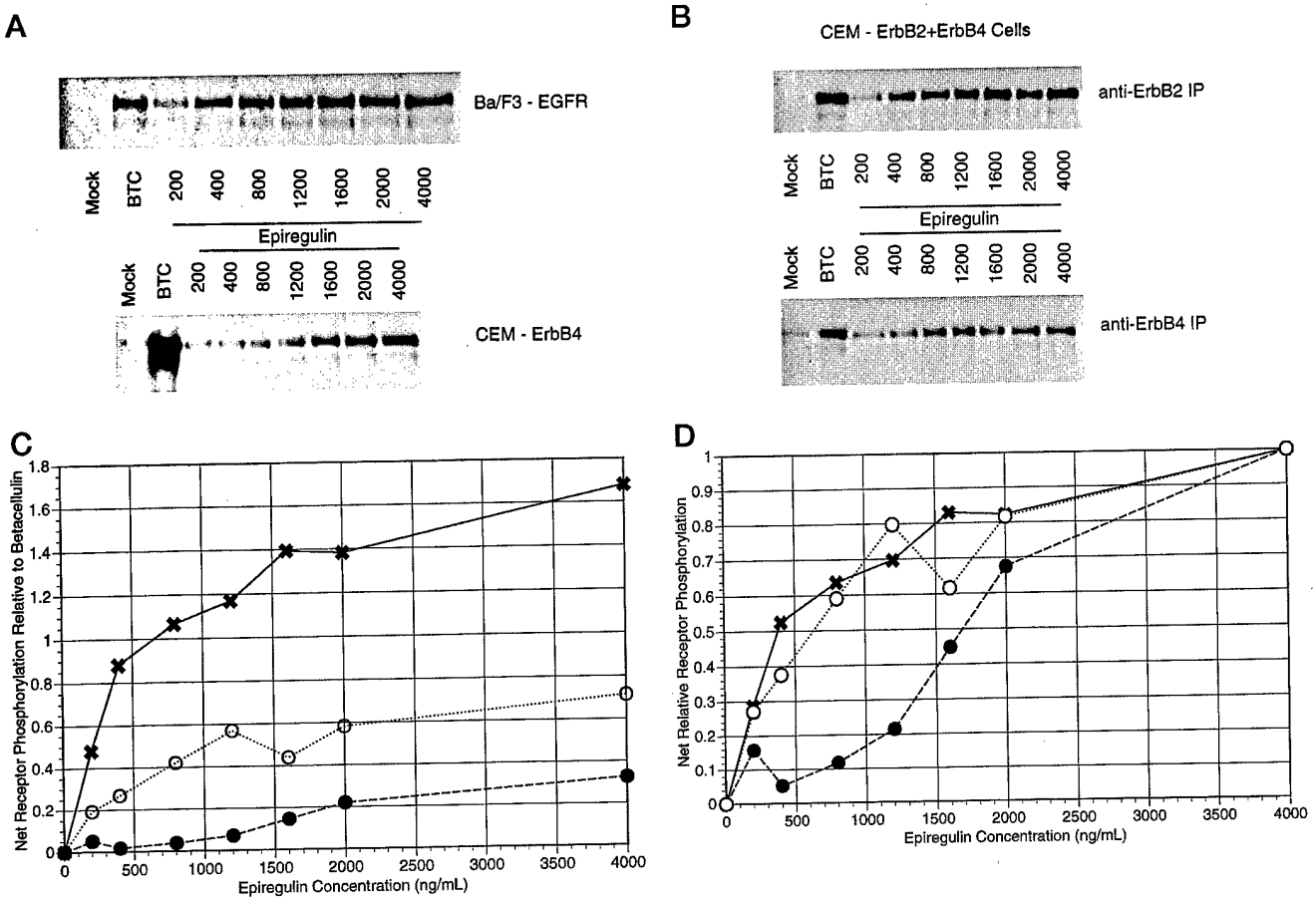

FIG. 5. **EPR dose response in BaF3 cells expressing EGFR or in CEM cells expressing ErbB4 alone or both ErbB2 and ErbB4.** *A* and *B*, BaF3/EGFR, CEM/ErbB4, or CEM/ErbB2+ErbB4 cells were stimulated with 100 ng/ml BTC (*BTC*), increasing concentrations of epiregulin as indicated or were mock stimulated with phosphate-buffered saline (*Mock*) as described previously (7, 14). EGFR, ErbB2, or ErbB4 was immunoprecipitated as indicated or appropriate using specific antireceptor antibodies and separated by SDS-PAGE as described previously (14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14). *C* and *D*, antiphosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3p flatbed scanner set for 600 dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation was either expressed relative to the tyrosine phosphorylation stimulated by 100 ng/ml BTC (*C*) or relative to the maximal receptor tyrosine phosphorylation stimulated by EPR (*D*). ✖, EGFR; ●, ErbB4; ○, 2+4 Anti-4.

TABLE I
Relative sensitivities of EGFR and ErbB4 to EPR and BTC
Data are adapted from Figs. 5, *A–D* and 6, *A–C*.

Cell line & receptor	EPR yielding half-maximal receptor activation	BTC yielding half-maximal receptor activation
BaF3/EGFR	380 ng/ml	NT[a]
CEM/ErbB4	1790 ng/ml	NT
BaF3/EGFR+ErbB4		
EGFR	320 ng/ml	35 ng/ml
ErbB4	790 ng/ml	5 ng/ml
CEM/ErbB2+ErbB4		
ErbB2	400 ng/ml	NT
ErbB4	630 ng/ml	NT

[a] NT, not tested.

ErbB4 homodimers.

The EPR and BTC Dose-Response Curves Are Different in Cells Expressing EGFR and ErbB4—EPR resembles BTC in its ability to activate either EGFR or ErbB4 when expressed individually (7). Yet, at saturation EPR stimulated almost 2-fold more EGFR phosphorylation than BTC, whereas BTC activated about 3-fold more ErbB4 phosphorylation than did EPR (Fig. 5, *A* and *C*). This suggested that BTC and EPR are functionally distinct. Hence, we compared EGFR and ErbB4 phosphorylation following stimulation with increasing concentrations of BTC or EPR in a BaF3 cell line that expresses both EGFR and ErbB4 (Fig. 6*A*).

We first compared the magnitude of receptor phosphorylation stimulated by EPR and BTC by plotting receptor phosphorylation relative to the maximal phosphorylation stimulated by BTC (Fig. 6, *B* and *C*). In agreement with results presented above (Fig. 5, *A* and *C*), EPR stimulated higher saturated levels of EGFR phosphorylation than BTC, whereas BTC activated greater ErbB4 phosphorylation than did EPR (Fig. 6, *A* and *C*). However, the magnitude of these differences was much less in the EGFR+ErbB4 cell line compared with the differences in phosphorylation that we observed between the cell lines expressing EGFR and ErbB4 individually (Fig. 5, *A* and *C*).

Next, we compared the sensitivities of EGFR and ErbB4 with BTC and EPR by identifying the growth factor concentrations required for half-maximal receptor phosphorylation. Half-maximal EGFR activation occurred at a BTC concentration of approximately 35 ng/ml, whereas half-maximal ErbB4 activation occurred at a BTC concentration of approximately 5 ng/ml (Fig. 6*B*; Table I). In contrast, half-maximal EGFR activation occurred at an EPR concentration of approximately 320 ng/ml, whereas half-maximal ErbB4 activation occurred at an EPR concentration of approximately 790 ng/ml (Fig. 6*C*; Table I).

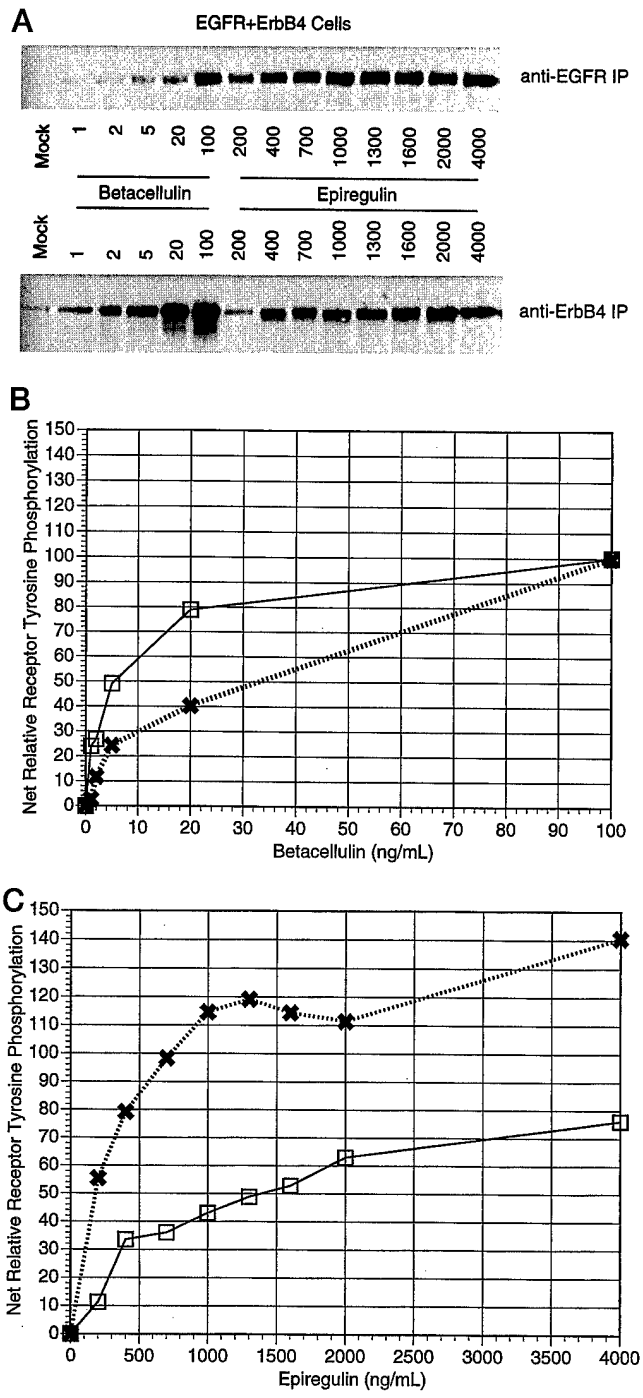

FIG. 6. **EPR and BTC dose response in BaF3 cells expressing both EGFR and ErbB4.** *A*, BaF3/EGFR+ErbB4 cells were stimulated with increasing concentrations of betacellulin or epiregulin or were mock stimulated with phosphate-buffered saline (*Mock*) as described previously (7, 14). EGFR or ErbB4 was immunoprecipitated as indicated using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14). *B* and *C*, antiphosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3p flatbed scanner set for 600 dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation stimulated by BTC (*B*) or EPR (*C*) was expressed relative to the maximal tyrosine phosphorylation stimulated by BTC. ✖, EGFR; □, ErbB4.

This suggests that ErbB4 is 7-fold more sensitive to BTC than is EGFR, whereas EGFR is more than 2-fold more sensitive to EPR than is ErbB4.

Finally, these results illustrate that EGFR expression, like ErbB2 expression, shifts the EPR dose-response curve in cells expressing ErbB4. Half-maximal ErbB4 phosphorylation in a CEM cell line expressing ErbB4 alone occurs at an EPR concentration of 1790 ng/ml (Fig. 5*D*; Table I). In contrast, half-maximal ErbB4 phosphorylation in BaF3 cells expressing both EGFR and ErbB4 occurs at 790 ng/ml (Fig. 6*C*; Table I).

EPR Activates ErbB Family Receptor Coupling to IL3 Independence—Although EPR and BTC stimulate qualitatively identical patterns of receptor phosphorylation, these hormones are quantitatively distinct. One possible mechanism is that EPR and BTC stimulate EGFR and ErbB4 tyrosine phosphorylation at different sites. This would account for the higher levels of EGFR activation by EPR compared with BTC and the higher levels of ErbB4 activation by BTC compared with EPR. Moreover, this would also enable these hormones to couple to distinct receptor effectors and physiologic responses. Therefore, we compared EPR and BTC induction of receptor coupling with physiologic responses. BaF3 cells require interleukin-3 (IL3) for survival and for proliferation. Activation of either EGFR or ErbB2 permits survival of BaF3 cells in the absence of IL3 (7, 14). However, ErbB4 activation by either NRG or BTC is not coupled to IL3-independent survival (7,14). In BaF3 cells expressing both ErbB2 and ErbB4 together, activation by either BTC or NRG induces IL3-independent survival, presumably through ErbB2 transmodulation by ErbB4 (7, 14).

EPR, like BTC (7), induces IL3-independent survival in BaF3 cells expressing EGFR, but not in vector control BaF3 cells or cells expressing ErbB2 (Fig. 7). (The IL3-independent response of BaF3 cells expressing ErbB2 to NRG is the result of ErbB2 transmodulation by endogenous ErbB3 in these cells (14).) EPR, BTC, and NRG all induced IL3 independence in cells co-expressing ErbB2 and ErbB4 (Fig. 7). This implies that BTC and NRG are functionally equivalent. However, the response to BTC and NRG is greater than the response to EPR, which may reflect subtle functional differences between BTC and EPR.

DISCUSSION

We previously demonstrated that the EGF family of peptide growth factors can be divided into three distinct functional groups (8) (Fig. 8). The first group consists of EGF, TGF-α, and amphiregulin. These hormones bind and activate only the EGFR, but they can activate the other three ErbB family receptors *in trans* via heterodimerization with the EGFR. The second group consists of NRG and NRG2, which bind ErbB3 and ErbB4 and transmodulate EGFR and Neu via the binding receptors. The third group consists of BTC, which binds and activates both EGFR and ErbB4. Recent data suggests that heparin-binding EGF-like growth factor may also bind and activate EGFR and ErbB4, which would make heparin-binding EGF-like growth factor a member of this group as well (22).

Although EPR activates both EGFR and ErbB4, the interactions of EPR with these two receptors appear to be quite different. Compared with BTC, EPR stimulates higher levels of EGFR phosphorylation and lower levels of ErbB4 phosphorylation. Whereas both EPR and BTC stimulate EGFR and ErbB4 homodimerization and signaling, the geometry of the receptor dimers induced by EPR and BTC may be subtly different. The alignment of the kinase domain of one receptor molecule of a receptor homodimer with the autophosphorylation site of the other receptor molecule following EPR stimulation could be different from this alignment following BTC stimulation, affecting the cross-phosphorylation within receptor dimers. Al-

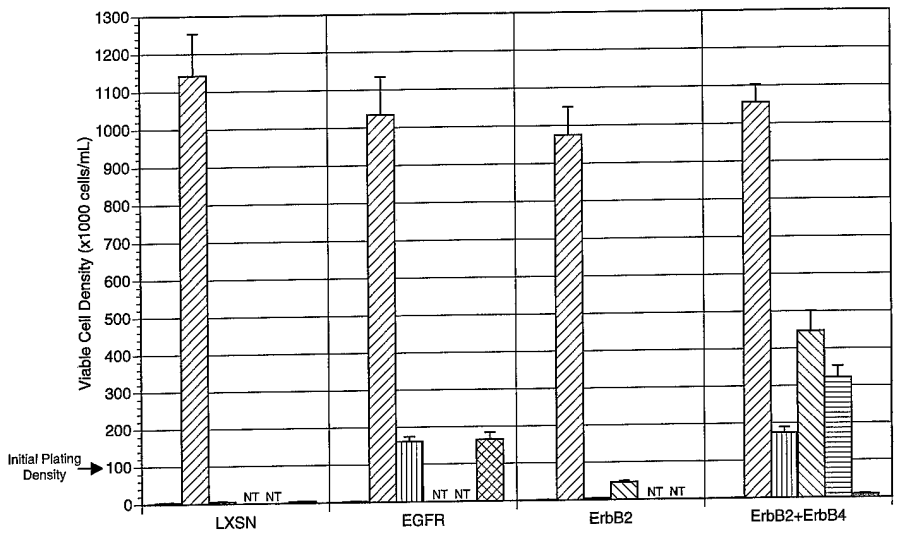

FIG. 7. **EPR stimulation of IL3-independent responses in BaF3 cells expressing various ErbB family receptors.** The IL3-independent responses of BaF3/LXSN (vector control), BaF3/EGFR, BaF3/ErbB2, and BaF3/ErbB2+ErbB4 cells to EPR stimulation were assayed as described earlier (7, 14). Briefly, cells were seeded in duplicate or triplicate at an initial density of 100×10^3 cells/ml in medium lacking IL3 (▨), containing IL3 (▨), or lacking IL3 but supplemented with 10 ng/ml EPR (▨), 10 ng/ml BTC (▨), 10 ng/ml synthetic NRGβ (▨) or 10 ng/ml TGF-α (▨). After seeding, samples were taken every 24 h, and the viable cell density was calculated by staining cells with trypan blue and counting them in a hemocytometer. Samples were taken until the viable cells reached a saturation density. The mean and standard error densities for three to seven trials are shown. *NT*, not tested. Unpublished work has demonstrated that 10 ng/ml BTC induces saturated amounts of IL3 independence in a variety of BaF3 cell lines, whereas 10 ng/ml EPR induces saturated amounts of IL3 independence in the BaF3/EGFR cell line.

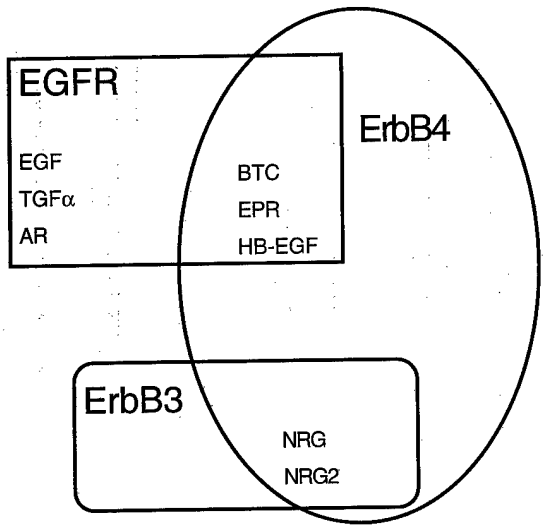

FIG. 8. **Venn diagram illustrating the activities of EGF family hormones.** The three functional groups of EGF family hormones are illustrated using a Venn diagram. The diagram is based on data presented in this work and data from Refs. 7, 8, 14, and 22.

ternatively, ligand-induced changes in the conformation of the receptor kinase domains might be different when the receptors are activated by BTC and EPR. Therefore, BTC and EPR may differentially stimulate receptor kinase activity. In either scenario, BTC and EPR could stimulate receptor autophosphorylation on different tyrosine residues, which could also be reflected in differences in gross levels of receptor phosphorylation. In this manner BTC and EPR could differentially modulate receptor coupling to signaling effectors and physiologic responses.

Another difference between EPR and BTC is that while EGFR is much more sensitive than ErbB4 to EPR, EGFR is less sensitive than ErbB4 to BTC. This suggests that although the affinity of EPR for EGFR is higher than the affinity for ErbB4, the affinity of BTC for EGFR is lower than the affinity for ErbB4. This too suggests that BTC and EPR have distinct biological functions, even in cells with identical patterns of ErbB family receptor expression.

Another important aspect of EPR function is the observation that the sensitivity of ErbB4 for EPR and the magnitude of ErbB4 activation by EPR can be modulated by the expression of other ErbB family receptors. EGFR expression increases the sensitivity of ErbB4 for EPR, suggesting that EGFR-ErbB4 heterodimers have a higher affinity for EPR than ErbB4-ErbB4 homodimers (Fig. 5, *A* and *D*; Fig. 6, *A* and *C*; Table I). Of course an alternative explanation is that the increased ErbB4 sensitivity in the presence of EGFR is due solely to EPR-induced transphosphorylation of ErbB4 by EGFR.

ErbB2 also increases the sensitivity of ErbB4 for EPR (Fig. 5, *A-D*; Table I). Because EPR does not activate ErbB2 in cells devoid of EGFR or ErbB4 (Figs. 1 and 7), the mechanism for the increased sensitivity of ErbB4 for EPR may be that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than do ErbB4-ErbB4 homodimers. These results resemble observations made with NRG, which does not bind to ErbB2, binds with low affinity to cells expressing ErbB3, and binds with higher affinity to cells that express both ErbB2 and ErbB3 (12). ErbB2 expression also increases the magnitude of ErbB4 activation by EPR.

These observations that ErbB4 activation by EPR can be influenced by EGFR or ErbB2 is consistent with existing models for receptor heterodimerization and transmodulation. It has been proposed that receptor heterodimerization is mediated through low affinity hormone-receptor interactions and heterotypic receptor-receptor contacts, after which there is cross-phosphorylation by the receptor kinase domains (23). It is possible that EGFR and ErbB2 are favored over ErbB4 for dimerization with ErbB4 in the presence of EPR. Therefore, there would be greater ErbB4 dimerization in cells expressing EGFR and ErbB4 or Neu and ErbB4 than in cells expressing ErbB4 alone. This may account for the increased sensitivity of ErbB4 for EPR in the presence of EGFR or ErbB2. It is also possible that ErbB2 is a better kinase for ErbB4 than ErbB4 itself. Consequently, ErbB2 may cross-phosphorylate more ErbB4 tyrosine residues in receptor heterodimers than ErbB4 would in receptor homodimers. Similarly, ErbB2 may phospho-

rylate the same tyrosine residues as ErbB4 to a greater extent than does ErbB4. Either of these last two possibilities would account for the increased tyrosine phosphorylation of ErbB4 by EPR in the presence of ErbB2.

As this manuscript was being prepared for submission, it was reported that radiolabeled EPR can be cross-linked to EGFR and ErbB4 in human breast tumor cell lines but not to ErbB2 or ErbB3. Furthermore, EPR stimulated high levels of EGFR and ErbB4 tyrosine phosphorylation and more modest levels of ErbB2 and ErbB3 tyrosine phosphorylation (24). Because the cell lines used in these studies express at least two and in some cases all four ErbB family receptors, some caution must be used in interpreting these results. Nonetheless, these data are entirely consistent with our findings that EGFR and ErbB4 are the receptors for EPR.

To date there have been only a few clues to EPR function. EPR transcripts are not detected in normal adult mouse liver, kidney, brain, spleen, testis, or skeletal muscles. However, low levels of EPR transcripts are detectable in adult mouse lung, smooth muscle, and heart, whereas more robust EPR transcription is observed in whole embryo RNA samples from 7-day-old mouse embryos (25).[3] This implies that EPR plays a significant role in early mammalian development but only a limited role in adult tissues.

Additional hints to EPR function arise from our data suggesting that EPR is a ligand for both EGFR and ErbB4. In most contexts EGFR activation is coupled to cellular DNA synthesis and proliferation. In contrast, there is mounting evidence that activated ErbB4 is coupled to growth inhibition, differentiation, and possibly tumor suppression. NRG, a ligand for ErbB3 and ErbB4, inhibits the proliferation and stimulates the differentiation of a number of human breast tumor cell lines (26), whereas NRG implants stimulate the differentiation of the mouse mammary epithelium *in vivo* (27). BTC stimulates the differentiation of pancreatic AR42J cells into insulin-secreting cells, but EGF and TGF-α do not (28). Finally, agonistic anti-ErbB4 antibodies stimulate the differentiation of human breast tumor cell lines (29), and ErbB4 overexpression in breast cancer patients correlates with progesterone receptor expression, which is a marker for longer disease-free survival and better overall prognosis (30). Because EPR is a ligand for both EGFR and ErbB4, EPR may act as a proliferative agent in cells expressing EGFR and may act as a differentiation agent in cells that express ErbB4.

Furthermore, because EPR activation of ErbB4 is regulated by ErbB2 and activated ErbB2 appears to couple to mitogenesis and cell proliferation, the effects of EPR on cells expressing ErbB4 may be tightly linked to a balance of ErbB4 and ErbB2 expression. In cells having relatively low levels of ErbB2, EPR may have little effect because it fails to bind to ErbB4, and in cells having moderate levels of ErbB2 and high levels of ErbB4, EPR may act as a differentiation agent and inhibit cell proliferation, because the relatively high levels of ErbB4 signaling may overcome the effects of ErbB2 signaling. Finally, in cells having relatively high levels of ErbB2 relative to ErbB4, EPR may stimulate such high levels of ErbB2 signaling that the effects of ErbB4 signaling are overcome, and cell proliferation is stimulated. In sum, our data suggests that the physiologic response to EPR will be dictated by relative levels of EGFR, ErbB2, and ErbB4 expression and not just the absolute level of expression of any single ErbB family receptor.

[3] T. Komurasaki, unpublished data.

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REFERENCES

1. Stern, D. F., Heffernan, P. A., and Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6,** 1729–1740
2. King, C. R., Borrello, I., Bellot, F., Comoglio, P., and Schlessinger, J. (1988) *EMBO J.* **6,** 1647–1651
3. Stern, D. F., and Kamps, M. P. (1988) *EMBO J.* **7,** 995–1001
4. Goldman, R., Ben-Levy, R., Peles, E., and Yarden, Y. (1990) *Biochemistry* **29,** 11024–11029
5. Wada, T., Qian, X., and Greene, M. I. (1990) *Cell* **61,** 1339–1347
6. Johnson, G. R., Kannan, B., Shoyab, M., and Stromberg, K. (1993) *J. Biol. Chem.* **268,** 2924–2931
7. Riese, D. J., II, Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996) *Oncogene* **12,** 345–353
8. Riese, D. J., II, Kim, E. D., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G. D., and Stern, D. F. (1996) *J. Biol. Chem.* **271,** 20047–20052
9. Culouscou, J.-M., Plowman, G. D., Carlton, G. W., Green, J. M., and Shoyab, M. (1993) *J. Biol. Chem.* **268,** 18407–18410
10. Plowman, G. D., Green, J. M., Culouscou, J.-M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) *Nature* **366,** 473–475
11. Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994) *J. Biol. Chem.* **269,** 14303–14306
12. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III. (1994) *J. Biol. Chem.* **269,** 14661–14665
13. Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, J., Yayon, A., Wen, D., and Yarden, Y. (1994) *J. Biol. Chem.* **269,** 25226–25233
14. Riese, D. J., II, van Raaij, T. M., Plowman, G. D, Andrews, G. C., and Stern, D. F. (1995) *Mol. Cell. Biol.* **15,** 5770–5776
15. Chang, H., Riese, D. J., II, Gilbert, W., Stern, D. F., and McMahan, U. J. (1997) *Nature* **387,** 509–512
16. Carraway, K. L., III, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M., and Lai, C. (1997) *Nature* **387,** 512–516
17. Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K. (1995) *J. Biol. Chem.* **270,** 7495–7500
18. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., III. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8132–8136
19. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996b) *EMBO J.* **15,** 2452–2467
20. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) *Mol. Cell. Biol.* **16,** 5276–5287
21. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997) *EMBO J.* **16,** 1647–1655
22. Elenius, K., Paul, S., Allison, G., Sun, J., and Klagsbrun, M. (1997) *EMBO J.* **16,** 1268–1278
23. Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D. M., and Schlessinger, J. (1997) *EMBO J.* **16,** 281–284
24. Komurasaki, T., Toyoda, H., Uchida, D., and Morimoto, S. (1997) *Oncogene* **15,** 2841–2848
25. Toyoda, H., Komurasaki, T., Ikeda, Y., Yoshimoto, M., Morimoto, S. (1995) *FEBS Lett.* **377,** 403–407
26. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R., and Yarden, Y. *Cell* **69,** 205–216
27. Jones, F. E., Jerry, D. J., Guarino, B. C., Andrews, G. C., and Stern, D. F. (1996) *Cell Growth Differ.* **7,** 1031–1038
28. Mashima, H., Ohnishi, H., Wakabayashi, K., Mine, T., Miyagawa, J.-I., Hanafusa, T., Seno, M., Yamada, H., and Kojima, I. (1996) *J. Clin. Invest.* **97,** 1647–1654
29. Chen, X., Levkowitz, G., Tzahar, E., Karunagaran, D., Lavi, S., Ben-Baruch, N., Leitner, O., Ratzkin, B. J., Bacus, S. S., and Yarden, Y. (1996) *J. Biol. Chem.* **271,** 7620–7629
30. Bacus, S. S., Chin, D., Yarden, Y., Zelnick, C. R., and Stern, D. F. (1996) *Am. J. Path.* **148,** 549–558
31. Palacios, R., and Steinmetz, M. (1985) *Cell* **41,** 727–734
32. Barbacci, E. G., Guarino, B. C., Stroh, J. G., Singleton, D. H., Rosnack, K. J., Moyer, J. D., and Andrews, G. C. (1995) *J. Biol. Chem.* **270,** 9585–9589
33. Gill, G. N., Kawamoto, T., Cochet, C. Le, A., Sato, J. D., Masui, H., McLeod, C., and Mendelsohn, J. (1984) *J. Biol. Chem.* **259,** 7755–7760